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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C07K 14/28, 14/245, A61P 37/02, A61K 39/108, 39/112, G01N 33/68

A1

(11) International Publication Number: **WO 00/14114**

(43) International Publication Date: 16 March 2000 (16.03 00)

(21) International Application Number: PCT/GB99/02970

(22) International Filing Date: 7 September 1999 (07.09.99)

(30) Priority Data:
9819484.8 7 September 1998 (07.09.98) GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG)

Published
With international search report
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PEPTIDE FRAGMENTS OF CHOLERA TOXIN B OR ENTEROTOXIN B AS VACCINE ADJUVANTS

(57) **Abstract**

A substance is described. The substance comprises any one or more of an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB; but wherein the substance does not exhibit GM-1 binding activity.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02970

A CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/28 C07K14/245 A61P37/02 A61K39/108 A61K39/112
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
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X	EP 0 095 426 A (CENTRE NAT RECH SCIENT ;PASTEUR INSTITUT (FR)) 30 November 1983 (1983-11-30) claim 7	1-3
A	DE 34 30 894 A (YEDA RES & DEV) 14 March 1985 (1985-03-14) claims; examples	1

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[X] Further documents are listed in the continuation of box C

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Date of the actual completion of the international search	Date of mailing of the international search report
8 February 2000	15/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/GB 99/02970

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PEPTIDE FRAGMENTS OF CHOLERA TOXIN B OR ENTEROTOXIN B AS VACCINE ADJUVANTS

FIELD OF THE INVENTION

The present invention relates to a substance.

In particular, the present invention relates to a substance that is capable of displaying one or more properties that are useful in medicine.

By way of example, the substance is useful for use as an immunomodulator and/or an adjuvant and/or an inhibitor of toxin-induced diarrhoea.

More in particular, the present invention relates to the use of an immunomodulatory substance in modulating an immune response - such as that associated with an autoimmune disease.

More in particular, the present invention relates to the use of the substance as an adjuvant when given in combination with a related or an unrelated antigen.

More in particular, the present invention relates to the use of the substance for inhibiting toxin-induced diarrhoea.

The present invention also relates to an assay for screening for agents capable of interacting with the substance of the present invention.

BACKGROUND OF THE INVENTION

Escherichia coli (E. coli) heat labile enterotoxin (Etx) and its closely related homologue, cholera toxin (Ctx) from *Vibrio cholerae,* are examples of protein toxins which bind to glycolipid receptors on host cell surfaces. Each toxin consists of six noncovalently linked polypeptide chains, including a single A subunit (27 kDa) and five identical B subunits (11.6 kDa) which bind to GM-1 ganglioside receptors found

on the surfaces of mammalian cells (Nashar *et al* 1996 Proc Natl Acad Sci 93: 226-230). The A subunit is responsible for toxicity possessing adenosine diphosphate (ADP) ADP-ribosyltransferase activity, whereas the B subunits (EtxB and CtxB) are non-toxic oligomers which bind and cross-link a ubiquitous cell surface glycolipid ganglioside, called GM-1, thus facilitating A subunit entry into the cell.

In contrast to the poor immunogenicity of the A subunit alone, both EtxB and CtxB are exceptionally potent immunogens and their respective holotoxins, Etx and Ctx (which comprise the A and B subunits) are known to be potent adjuvants when given orally in combination with unrelated antigens (Ruedl *et al* 1996 Vaccine 14: 792-798; Nashar *et al* 1993 Vaccine 11: 235; Nashar and Hirst 1995 Vaccine 13: 803; Elson and Ealding 1984 J Immunol 133: 2892; Lycke and Holmgren 1986 Immunology 59: 301). Because of their immunogenicity, both EtxB and CtxB have been used as carriers for other epitopes and antigens (Nashar *et al* 1993 *ibid*) and have been used as components of vaccines against cholera and *E.coli* mediated diarrhoeal diseases (Jetborn *et al* 1992 Vaccine 10: 130).

Several studies have been carried out on the immunodominant epitope of the CtxB and EtxB subunits with a view to developing a vaccine against the cholera toxin and heat labile *E.coli* toxin. By way of example, the following disclosures represent some of the work that has been carried out in this area.

UK Patent Application No. 2 415 419A discloses a synthetic vaccine against cholera and against heat labile toxin of *E. coli* comprising a conjugate of a carrier with a synthetic polypeptide corresponding to part of the sequence of CtxB.

WO 85/02611 discloses synthetic polypeptides corresponding to particular sequences of EtxB which are deemed useful as conjugates or as an active ingredient to raise antibodies against the B subunit and for protecting a host animal against infection by enterotoxins.

WO 89/10967 discloses an amino acid sequence which represents residue numbers 50-64 of the CtxB which can be used in combination with an epitopes of a heterologous organism, such as *Flagellum* and/or *Salmonella,* in vaccine formulations with a view to providing protection against infection by the heterologous organism or to providing protection against conditions or disorders caused by an antigen of the organism.

WO 90/03437 relates to a hybrid protein which fuses the CtxB subunit with the active sequence of a heterologous antigen which is deemed useful for vaccination purposes, particularly to help the stabilisation of heterologous antigens in the intestinal environment.

WO 94/06465 relates to amino acid fragments which are linked, either with or without a linker, to an appropriate carrier such that the amino acid fragment linked to the carrier generates an opsonic or protective immune response to the epitopes of the fragment.

WO95/29701 discloses a vaccine against *Vibrio cholera* which comprises a conjugate of cholera toxin B subunit (CTB) or a synthetic fragment peptide which consists of a portion thereof, such as peptide CTP 3 comprising the 50-64 amino acid sequence of the B chain linked to an inert carrier.

WO96/26282 discloses an expression systems for expressing gene products from recombinant *Bordetella* strains wherein the gene product may be a cholera toxin molecule.

WO96/34893 discloses hybrid molecules between EtxB and CtxB which may be useful as a vaccine and to prevent and/or treat enterotoxin-induced illness in an individual.

WO 98/21344 discloses an EtxB subunit which is modified to include an inserted antigenic peptide. The chimeric antigen-EtxB molecule is used to elicit an antibody response against an antigenic peptide in host animals.

These studies related to either the use of (i) a peptide comprising part of the sequence of CtxB/EtxB or (ii) a peptide comprising part of the sequence of CtxB/EtxB coupled to a second entity (such as an antigen) to induce and/or maximise the immunological response to that peptide. Accordingly, these documents relate to the use of an immunodominant epitope of CtxB/EtxB or parts thereof as an immunogen in inducing an immunological response against these subunits with a view to developing immunity against cholera and/or *E.coli* mediated diarrhoea diseases. WO 91/07979 discloses a chimeric protein which includes a portion of CtxB and an epitope region of a desired antigen which are designed for use as a vaccine to elicit an immune response in a subject to a desired antigen. In this regard, the portion of CtxB is being used as an adjuvant but not as an immunomodulator. Accordingly, none of the above cited documents relates to the use of a CtxB/EtxB peptide or part thereof as an immunotherapeutic capable of modulating the immune response.

We have shown that the EtxB subunit is capable of acting as an immunomodulator in immune disorders. By way of example, we have disclosed in WO 97/02045 that EtxB binds to GM-1 ganglioside receptors which are found on the surfaces of mammalian cells and that this binding induces differential effects on lymphocyte populations including a specific depletion of CD8+ T cells and an associated activation of B cells. These effects are absent when a mutant EtxB protein (G33D) (lacking GM-1 binding activity) is employed. Consequently, these experimental results would suggest that all of the functionalities associated with EtxB and CtxB are attributable to the capacity of the EtxB and CtxB subunits to bind to the GM-1 receptor since mutants lacking the capacity to bind GM-1 (such as EtxB (G33D)) fail to act as adjuvants or immunomodulators. Thus, the prior art to date has suggested that immunomodulation and other effects of Etx and Ctx are mediated through GM-1 binding. However, until now, no investigations have been carried out on CtxB/EtxB mutants which retain the capacity to bind to the GM-1 receptor but which lack an immunomodulatory effect.

We have suprisingly found that not all of the effects of Etx and Ctx are mediated through GM-1 binding.

SUMMARY ASPECTS OF THE INVENTION

In accordance with the present invention we have now found that the immunomodulation and some other effects of Etx and Ctx are not mediated through GM-1 binding.

Aspects of the present invention are presented in the accompanying claims and in the following description and discussion.

In one aspect of the present invention there is provided a substance comprising any one or more of: an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB; but wherein the substance is not capable of exhibiting GM-1 binding activity.

In a preferred aspect of the present invention there is provided a substance comprising any one or more of: an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to loop of EtxB and/or CtxB; but wherein the substance does not exhibit GM-1 binding activity.

In a highly preferred aspect of the present invention there is provided a substance comprising any one or more of: an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to the $\beta 4$-$\alpha 2$ loop of EtxB and/or CtxB; but wherein the substance does not exhibit GM-1 binding activity.

The substance of the present invention can be an amino acid sequence or a chemical derivative thereof. The substance may be a synthetic peptide or a synthetic peptide variation - such as a retroinverso D peptide. The substance may even be an organic compound or other chemical. The latter examples are example of mimetics of SEQ ID No. 2.

The susbstance of the present invention is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB.

The term "same as or is similar to" is a qualitative term rather than a quantitative term. In this respect, it may be desirable to have an increased binding affinity.

An assay for determining whether a substance is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB would be readily determinable to those skilled in the art. For example, the assay may measure and/or determine an effect on cell populations, such as lymphocyte cell populations. These effects can include but are not limited to an induction of apotosis in CD8+ T cells , the enhanced activation of CD4+ T cells and the polyclonal activation of B cells. In addition, or in the alternative, the assay could be based on determining and/or measuring particular cell surface marker(s) indicative of activation of certain intracellular events (e.g. meauring an increase in CD25 expression).

The susbstance of the present invention is not capable of exhibiting GM-1 binding activity.

An assay for determining the lack of GM-1 binding activity would be readily determinable to those skilled in the art. For example, the assay may utilise GM-1 bound to a solid support and wherein the substance is then passed across the bound GM-1. Elution of the substance is indicative that it does not bind to GM-1. In a more preferred aspect, the assay is that described in WO 97/02045.

It is to be noted that the binding activity of the substance is not necessarily dependent on a primary binding event as is found with full length Ctx and EtxB subunits. With full length Ctx and EtxB, the primary binding activity is GM-1 binding activity. In this regard, the substance may exhibit a single binding event. However, for some cases, the substance may possess the capability of having more than one binding activity.

Preferably, the substance is substantially isolated and/or substantially pure.

As used herein, the terms "isolated" and "purified" refer to molecules, either nucleic or amino acid sequences, that are removed from their natural environment and/or isolated or separated from at least one other component with which they are naturally associated. A protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the substance and still be regarded as substantially isolated.

The present invention is based on the suprising finding that there are mutants which are capable of binding to the GM-1 receptor but which lack an immunomodulatory effect. These mutants facilitate the elucidatation of the mechanism by which the B subunits of Ctx and/or Etx act, particularly *vis-a-vis* an immunomodulatory effect.

Other aspects of the present invention are as follows.

A substance according to the present invention for use in medicine.

A substance according to the present invention for use as an immunomodulator.

A substance according to the present invention for use as an adjuvant.

A substance according to the present invention for use as an inhibitor of toxin-induced diarrhoea.

A substance according to the present invention wherein the substance additionally comprises an antigen or an antigenic determinant.

A pharmaceutical composition comprising the substance according to the present invention, optionally admixed with one or more pharmaceutically acceptable carrier(s), diluent(s) or excipient(s).

Use of a substance according to the present invention for use in the manufacture of a medicament that is capable of treating and/or preventing and/or modulating a disease and/or condition associated with an immune disorder and/or a toxin mediated disorder.

An assay method for determing one or agents that are capable of interacting with and/or affecting the substance according to the present invention; wherein the assay comprises contacting the substance with an agent to be tested, and then determining whether or not the agent affects the substance.

An agent identified by the assay method according to the present invention.

A method of treatment, comprising administering to a subject in need of treatment of and/or prevention of and/or modulation of a disease and/or condition associated with an immune disorder and/or a toxin mediated disorder a substance according to the present invention.

These aspects are presented under separate section headings. However, it is to be understood that the teachings under each section heading are not necessarily limited to that particular section heading.

IMMUNOMODULATOR

As used herein, the term "immunomodulator" means a substance that is capable of modulating the immune response by inducing, for example, a differential effect on cells, such as lymphocyte cells - preferably leading to induction of apoptosis in CD8+ T cells and/or enhanced activation of CD4+ cells and/or the polyclonal activation fo B cells.

The term "differential effect on leukocyte cells" may include but is not limited to a specific depletion of CD8+ cells (through for example apotosis), the enhanced activation of CD4+ T cells and/or an associated activation of B cells.

Preferably the immunomodulator is capable of downregulating the pathological response of Th1 and/or Th2-associated immune responses.

Preferably the immunomodulator is capable of upregulating the production of antibodies at mucosal surfaces.

ADJUVANT

As used herein, an "adjuvant" is a substance which non-specifically enhances the immune response to an antigen.

It also includes any substance which is capable of affecting the extent of the immune response to an entity such as an antigen and/or an antigenic determinant, by altering the antigenicity of the antigen or by altering the specific reactivity or the nonspecific effector associated mechanisms of the host such that an immune response is induced in a host cell and/or is guided in a particular direction. In one preferred aspect, the adjuvant is capable of acting as a mucosal adjuvant.

Preferably the adjuvant is capable of prolonging antigen presentation and providing a sustained immunologic memory in a mammalian subject.

ANTIGEN

As used herein, an "antigen" means an entity which, when introduced into an immunocompetent host, stimulates the production of a specific antibody or antibodies that can combine with the entity. The antigen may be a pure substance, a mixture of substance or soluble or particulate material (including cells or cell fragments). In this sense, the term includes any suitable antigenic determinant, auto-antigen, self-antigen, cross reacting antigen, alloantigen, xenoantigen, tolerogen, allergen, hapten, and immunogen, or parts thereof, as well as any combination thereof, and these terms are used interchangeably throughout the text.

An "allergen" includes any antigen that stimulates an allergic reaction, inducing a Type I hypersensitivity reaction.

Examples of common allergen sources include but are not limited to ragweed, rye, couch, wild oat, timothy, Bermuda, Kentucky blue, mugwortalder, birch, hazel, beech, Cupressae, oak, olive, *Aspergillus* spp., *Cladosporium* spp., *Alternaria* spp., Basidospores, Ascomyceteswheat, rye, oatcat, dog, horse, rabbit, guinea pig, hamsterbudgerigar, parrot, pigeon, duck, chicken, Dermatophagoides pteronyssinus, D.farinae, Euroglyphus maynei, cockroach, fly, locust, midge, seafood, legumes, peanuts, nuts, cereals, dairy products, eggs, fruits, tomatoes, mushrooms, alcoholic beverages, coffee, chocolate, penicillins, sulphonamides and other antibiotics, sulphasalazine, carbamazepine, bee and wasp stings, ant and mosquito bitesblood products, sera, vaccines, contrast media, drugs (including anti-asthma drugs and antibiotics).

ANTIGENIC DETERMINANT

The term "antigenic determinant" as used herein refers to a site on an antigen which is recognised by an antibody or T-cell receptor. Preferably it is a short peptide derived from or as part of a protein antigen. However the term is also intended to include glycopeptides and carbohydrate epitopes. The term also includes modified sequences

of amino acids or carbohydrates which stimulate responses which recognise the whole organism.

It is advantageous if the antigenic determinant is an antigenic determinant of the infectious agent (such as a bacterium or virus) which causes the infectious disease.

By way of example, if the infectious agent is EBV, the antigenic determinant may be an antigenic determinant of gp340 or gp350 or of a latent protein (for example EBNAs 1,2 3A, 3B, 3C and -LP, LMP-1, -2A and 2B or an EBER). If the infectious agent is an influenza virus, the antigenic determinant may be an antigenic determinant of a viral coat protein (for example haemagglutinin and neuraminidase) or of an internal protein (for example, nucleoprotein). If the infectious agent is selected from the group consisting of enteropathogenic, enterotoxigenic, enteroinvasive, enterohaemorrhagic and enteroaggregative *E.coli*, then the antigenic determinant may be an antigenic determinant of a bacterial toxin or adhesion factor.

It is also advantageous if the antigenic determinant is an antigenic determinant from an autoantigen.

AGENT

The agent can be an amino acid sequence or a chemical derivative thereof. The substance may even be an organic compound or other chemical. The agent may be a nucleotide sequences - which may be sense or anti-sense sequences. The agent may be an antibody. In one preferred aspect, the agent is a cell receptor that is engageable by the substance.

INHIBITOR OF TOXIN-INDUCED DIARRHOEA

The term "inhibitor of toxin-induced diarrhoea" includes any substance which is capable of affecting the activity of Etx/Ctx holotoxins such that the pathological consequences of Etx/Ctx, such as diarrhoea, may be avoided.

DETAILED DESCRIPTION OF THE INVENTION

The present invention demonstrates the highly surprising finding that:

(i) the substance of the present invention is capable of acting as an immunomodulator and/or an adjuvant and/or an inhibitor of toxin-induced diarrhoea which is capable of affecting enterotoxin mediated diarrhoeal diseases.

(ii) the substance of the present invention is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB. The activity of the substance of the present invention may be mediated by the "so-called" ß4-α2 loop of EtxB and CtxB, which is a flexible loop included within amino acid residues 45-65.

(iii) EtxB molecules with point mutations at three separate sites within the ß4-α2 loop (positions 51, 56 and 57) retain GM-1 binding activity, but lack other activities, such as toxicity and the capacity to upregulate CD25 and trigger apoptosis of CD8-positive T-cells. In addition, Ctx holotoxins comprising B subunits with mutations also show a defect in an ability to trigger electrogenic chloride secretion, the primary secretory event responsible for mediating diarrhorea. These finding are particularly surprising, since flexible loops are usually thought to serve only to join two elements of secondary structure together, and rarely have an important function themselves.

(iv) the binding activity of the substance is not necessarily dependent on a primary binding event as is found with full length Ctx and EtxB. With full length Ctx and EtxB, the primary binding activity is GM-1 binding activity.

Ctx/Etx TOXINS

As used herein, the term "Ctx" refers to the cholera toxin and the term "CtxB" refers to the B subunit of the cholera toxin. In other texts, these may sometimes be identified as CT or Ct or CTB or CtB respectively.

As used herein, the term "Etx" herein means the *E. coli* heat labile enterotoxin and the term "EtxB" is the B subunit of Etx. In other texts, these may sometimes be identified as LT or Lt and LTB or LtB respectively.

ß4-α2 LOOP

In one aspect, the present invention relates to an substance comprising the sequence EVPGSQH (SEQ ID No 2) which is capable of acting in a manner that is the same or is similiar to EtxB and/or CtxB or a variant thereof, or a homologue thereof, or a fragment thereof or a derivative thereof or a mimetic thereof but which is not capable of exhibiting GM-1 binding activity.

Without wishing to be bound by theory, we believe that the binding of the five Etx/Ctx B subunits to GM-1 is a high affinity interaction, which allows a relatively low affinity secondary binding activity of EtxB/CtxB to occur. This binding is mediated by the the ß4-α2 loop of EtxB/CtxB. The structure of the ß4-α2 loop of EtxB/CtxB can be understood by reference to the molecular structure of Etx as described in detail in Sixma *et al.* J. Mol. Biol. (1993) 230; 890-918) and as illustrated in Figure 1.

In summary, each B subunit of Etx or Ctx consists of a small N-terminal helix (α1), two three-stranded anti-parallel sheets (sheet I, composed of strands ß2, ß3, ß4 and sheet II, composed of strands ß1, ß5 and ß6), and a long α-helix (α2). The two ß sheets form a ß barrel. The loops joining these elements of secondary structure in the B subunit can be divided into two classes, referring to the two ends of the sheets. On one end of the subunit, the "narrow" (or "A") end, the loops are generally short,

involving the connections ß1-ß2, ß3-ß4, and α2-ß5 as well as the C-terminus. The subunit widens at the other end, with much longer loops connecting secondary structure elements α1-ß1, ß2-ß3, ß4-α2. The longest loop connects ß4 and α2 (hereinafter the "ß4-α2 loop"), includes the residues Glu 51 to Asp 59, and extends below the plane of the ß sheets. This loop is quite flexible, but according to Sixma *et al* (Nature (1992) **355**; 561-564) becomes distinctly less mobile after lactose binding. The present invention demonstrates that the ß4-α2 loop of EtxB/CtxB is responsible for the secondary binding activity and so the use of this loop in isolation from the rest of the EtxB/CtxB molecule (for example as a peptide), may permit the secondary binding activity to occur in the absence of the first. Selective mutation of the ß4-α2 loop, or a peptide derived from this loop, may be exploited with a view to increasing the affinity of the secondary binding activity. By increasing the affinity of the secondary binding activity, the interaction with GM-1 may be further obviated.

As used herein, the term "ß4-α2 loop of EtxB/CtxB" is the entity which is responsible for the secondary binding activity of the B subunits of toxins such as the cholera toxin and heat labile *E.coli* toxin. When the ß4-α2 loop is used in isolation from the rest of the EtxB and/or CtxB molecule (for example as a peptide), the secondary binding activity may occur in the absence of the first and is herein after referred to as an activity or binding activity.

Preferably the substance of the present invention comprises an isolated ß4-α2 loop of EtxB/CtxB.

Preferably the substance comprises a mimetic of the isolated ß4-α2 loop of EtxB/CtxB.

Preferably the substance comprises a mimetic of the isolated ß4-α2 loop of EtxB/CtxB with a high affinity binding activity.

Preferably the substance comprise a peptide of from about 5 to about 40 amino acids.

Preferably the peptide has less than 25 amino acids.

If the peptide is a fusion protein, preferably the peptide has greater than 25 amino acids.

Preferably the substance comprises the sequence VEVPGSQHIDSQ (SEQ ID No 3).

Preferably the substance comprises the sequence GATFQVEVPGSQHIDSQKKAI (SEQ ID No 4).

Preferably the substance comprises the sequence GETFQVEVPGSQHIDSQKKAI (SEQ ID No 5) derivable from residues 45-65 of porcine *E.coli.*

Preferably the substance comprises residues 45-65 derivable from EtxB of the human variant of *E.coli* derivable from EtxB of the porcine variant of *E.coli.*

AMINO ACID SEQUENCE

The present invention provides a substance comprising the amino acid sequences of the present invention which is capable of acting as an immunomodulator and/or an adjuvant and/or an an inhibitor of toxin-induced diarrhoea which is capable of affecting enterotoxin mediated diarrhoeal diseases. The substance may also be used in assays for the identification of one or more agents capable of interacting with and/or affecting the substance activity.

As used herein, the term "amino acid sequence" refers to peptide, polypeptide sequences, protein sequences or portions thereof.

AFFECT

The term "affect" includes modulation, such as treatment, prevention, suppression, alleviation, restoration, elevation, modification of the substance activity.

The term "modification" includes but is not limited to disabling, silencing, mutating, removing, enhancing, increasing, agonising, antagonising, decreasing or blocking the substance activity.

VARIANTS/HOMOLOGUES/DERIVATIVES

Preferred amino acid sequences of the invention are SEQ ID No 2 or SEQ ID No 3 or SEQ ID No 4 or SEQ ID No 5 or sequences obtainable from the substance of the present invention but also include homologous sequences obtained from any source, for example related viral/bacterial proteins, cellular homologues and synthetic peptides, as well as variants or derivatives thereof.

Thus, the present invention covers variants, homologues or derivatives of the amino acid sequences presented herein, as well as variants, homologues or derivatives of the nucleotide sequence coding for those amino acid sequences.

In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least the 7 amino acids of SEQ ID No 2, for example as shown in the sequence listing herein. In particular, homology should typically be considered with respect to those regions of the sequence (such as amino acids at positions 51, 56 and 57) known to be essential for an activity which is the same or is similiar to EtxB and/or CtxB rather than non-essential neighbouring sequences. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The terms "variant" or "derivative" in relation to the amino acid sequences of the present invention presented as SEQ ID No 2, SEQ ID No 3, SEQ ID No 4 and SEQ ID No 5 includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant entity retains an activity, preferably having at least the same and/or similiar activity as CtxB and/or EtxB.

SEQ ID No 2 or SEQ ID No 3 or SEQ ID No 4 or SEQ ID No 5 may be modified for use in the present invention. Typically, modifications are made that maintain the activity of the sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the activity.

The substance of the present invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

NUCLEOTIDE SEQUENCE

In one aspect, the present invention provides nucleotide sequences encoding the substance of the present invention capable of acting as a template or as targets in assays (such as a yeast two hybrid assay) for the identification of one or more agents and/or derivatives thereof capable of affecting the substance.

As used herein, the term "nucleotide sequence" refers to nucleotide sequences, oligonucleotide sequences, polynucleotide sequences and variants, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be DNA or RNA of genomic or synthetic or recombinant origin which may be double-stranded or single-stranded whether representing the sense or antisense strand or combinations thereof. Preferably, the term nucleotide sequence is prepared by use of recombinant DNA techniques (e.g. recombinant DNA).

Preferably, the term "nucleotide sequence" means DNA.

The substance encoding nucleotide sequence may be the same as the naturally occuring form for this aspect. Preferably the nucleotide sequence encoding the substance is a non-native nucleotide sequence - or is a variant, homologue, fragment or derivative thereof. Thus, in a preferred embodiment, the present invention does not cover the native nucleotide coding sequence according to the present invention in its natural environment when it is under the control of its native promoter which is also in its natural environment. For ease of reference, we have called this preferred embodiment the "non-native nucleotide sequence".

As used herein "naturally occurring" refers to an substance with an amino acid sequence found in nature.

As used herein "biologically active" refers to an substance having regulatory or biochemical functions of the naturally occurring substance.

VARIANTS/HOMOLOGUES/DERIVATIVES

The terms "variant", "homologue" or "derivative" in relation to the nucleotide sequence SEQ ID No 1 of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for an substance having an activity, preferably having at least the same activity as the SEQ SEQ ID No 2, SEQ ID No 3, SEQ ID No 4 and SEQ ID No 5 presented in the sequence listings.

As indicated above, with respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the sequence listing herein. More preferably there is at least 95%, more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as described above. A preferred sequence comparison program is the GCG Wisconsin Bestfit program described above. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

The present invention also encompasses nucleotide sequences that are capable of hybridising selectively to the sequences presented herein, or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40 or 50 nucleotides in length.

As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

As used herein an "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring substance.

As used herein "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

HYBRIDISATION

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

Nucleotide sequences of the invention capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 75%, preferably at least 85 or 90% and more preferably at least 95% or 98% homologous to the corresponding nucleotide sequences presented herein over a region of at least 20, preferably at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides. Preferred nucleotide sequences of the invention will comprise regions homologous to nucleotides comprising SEQ ID No 1 preferably at least 80 or 90% and more preferably at least 95% homologous to SEQ ID No 1.

The term "selectively hybridizable" means that the nucleotide sequence used as a probe is used under conditions where a target nucleotide sequence of the invention is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other nucleotide sequences present, for example, in the cDNA or genomic DNA library being screened. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P.

Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular

Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related nucleotide sequences.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention under stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na_3 Citrate pH 7.0). Where the nucleotide sequence of the invention is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present invention. Where the nucleotide sequence is single-stranded, it is to be understood that the complementary sequence of that nucleotide sequence is also included within the scope of the present invention.

EXPRESSION VECTORS

The nucleotide sequences of the present invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence in and/or from a compatible host cell. Expression may be controlled using control sequences which include promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

The substance produced by a host recombinant cell may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The substance coding sequences can be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

FUSION PROTEINS

The substance of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the substance comprising the amino acid sequence of the present invention.

In one embodiment of the present invention, the fusion protein comprises an antigen or an antigenic determinant fused to the substance of the present invention. In this embodiment, the fusion protein is a non-naturally occuring fusion protein comprising a substance which may act as an adjuvant in the sense of providing a generalised stimulation of the immune system. The antigen or antigenic determinant may be attached to either the amino or carboxy terminus of the substance.

In another embodiment of the invention, the substance of the invention may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognized by a commercially available antibody.

In yet another embodiment, an assay for identifying an agent, such as a target receptor, or for an agent capable of regulating CD25 transcriptional activity may be conducted using a bound fusion protein.

ANTIBODIES

In one embodiment of the present invention, the substance of the present invention may be an antibody. This antibody may be capable of acting as a mimetic of the present invention.

Antibodies may be produced by standard techniques, such as by immunisation with the substance of the invention or by using a phage display library.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes but is not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library. Such fragments include fragments of whole antibodies which retain their binding activity for a target substance, Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv), fusion proteins and other synthetic proteins which comprise the antigen-binding site of the antibody. Furthermore, the antibodies and fragments thereof may be humanised antibodies, for example as described in substance-A-239400. Neutralizing antibodies, i.e., those which inhibit biological activity of the substance polypeptides, are especially preferred for diagnostics and therapeutics.

In one embodiment, the invention also provides monoclonal or polyclonal antibodies to substances of the invention such as polypeptides or fragments thereof. Thus, the present invention further provides a process for the production of monoclonal or polyclonal antibodies to substances, such as polypeptides of the invention.

POLYCLONAL ANTIBODIES

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing a epitope(s) obtainable from an identifed agent and/or substance of the present invention. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium parvum* are potentially useful human adjuvants which may be employed if purified the substance polypeptide is administered to immunologically compromised individuals for the purpose of stimulating systemic defence.

Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an epitope obtainable from an identifed agent and/or substance of the present invention contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use as immunogens in animals or humans.

MONOCLONAL ANTIBODIES

Monoclonal antibodies directed against epitopes obtainable from an identifed agent and/or substance of the present invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of

monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

Monoclonal antibodies to the substance and/or identified agent of the present invention may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975 Nature 256:495-497), the human B-cell hybridoma technique (Kosbor *et al* (1983) Immunol Today 4:72; Cote *et al* (1983) Proc Natl Acad Sci 80:2026-2030) and the EBV-hybridoma technique (Cole *et al* (1985) Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, pp 77-96). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al* (1984) Proc Natl Acad Sci 81:6851-6855; Neuberger *et al* (1984) Nature 312:604-608; Takeda *et al* (1985) Nature 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US Patent No. 4,946,779) can be adapted to produce the substance specific single chain antibodies.

Antibodies, both monoclonal and polyclonal, which are directed against epitopes obtainable from an identifed agent and/or substance of the present invention are particularly useful in diagnosis, and those which are neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies. Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the substance and/or agent against which protection is desired. Techniques for raising anti-idiotype antibodies are known in the art. These anti-idiotype antibodies may also be useful in therapy.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al* (1989, Proc Natl Acad Sci 86: 3833-3837), and Winter G and Milstein C (1991; Nature 349:293-299).

Antibody fragments which contain specific binding sites for the substance may also be generated. For example, such fragments include, but are not limited to, the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse WD *et al* (1989) Science 256:1275-128 1).

ASSAYS FOR IMMUNOMODULATORY SUBSTANCES

The immunomodulation of the immune response may be measured by transcriptional profiling, for example, by assaying for activation of transcription of CD25 cell surface marker by measuring the signal from a linked reporter gene.

REPORTERS

Preferably a wide variety of reporters may be used in the assay methods of the present invention with preferred reporters providing conveniently detectable signals (eg. by spectroscopy). By way of example, a reporter gene may encode an enzyme which catalyses a reaction which alters light absorption properties.

Examples of reporter molecules include but are not limited to β-galactosidase, invertase, green fluorescent protein, luciferase, chloramphenicol, acetyltransferase, β-glucuronidase, exo-glucanase and glucoamylase. Alternatively, radiolabeled or fluorescent tag-labeled nucleotides can be incorporated into nascent transcripts which are then identified when bound to oligonucleotide probes.

In one preferred embodiment, the production of the reporter molecule is measured by the enzymatic activity of the reporter gene product, such as β-galactosidase.

ASSAYS FOR INHIBITORS OF TOXIN-INDUCED DIARRHOEA

The substance of the present invention or a derivative or homologue thereof and/or a cell line that expresses the substance of the present invention or a derivative or homologue thereof may be used to screen for agents (such as antibodies, peptides, organic or inorganic molecules) capable of affecting the activity of the substance. By way of example, any agent capable of inhibiting the activity of the substance may be screened for inhibitors of toxin-induced diarrhoea thereby identifying agents capable of affecting the cholera and/or enterotoxin mediated diarrhoeal diseases.

In one embodiment, the screens of the present invention may identify antagonists of the substance of the present invention, such as antibodies, peptides or small organic molecules which are capable of acting as inhibitors of toxin-induced diarrhoea

ASSAYS FOR AGENTS

Phage display may be employed in the identification of agents, such as a cell surface receptor that is engageable by the substance of the present invention. The positive identification of such a receptor may faciliate the use of combinatorial libraries to identify mimetics capable of acting in the same or a similiar manner as the substance of the present invention.

Phage display is a protocol of molecular screening which utilises recombinant bacteriophage. The technology involves transforming bacteriophage with a gene that encodes an appropriate ligand (in this case a candidate agent) capable of reacting with a target substance (or a derivative or homologue thereof) or the nucleotide sequence (or a derivative or homologue thereof) encoding same. The transformed bacteriophage (which preferably is tethered to a solid support) expresses the appropriate ligand (such as the candidate agent) and displays it on their phage coat. The entity or entities (such as cells) bearing the target substance molecules which recognises the candidate agent are isolated and amplified. The successful candidate agents are then characterised. Phage display has advantages over standard affinity

ligand screening technologies. The phage surface displays the candidate agent in a three dimensional configuration, more closely resembling its naturally occuring conformation. This allows for more specific and higher affinity binding for screening purposes.

ASSAYS FOR MIMETICS

In one embodiment, the screens of the present invention may identify mimetics of the substance of the present invention, such as antibodies, or other chemical compounds which have an immunomodulatory and/or adjuvant effect.

Such mimetics can be administered alone or in combination with other therapeutics for the treatment of diseases of the present invention.

SCREENS

The substance of the present invention to be used for identifying immunomodulators, adjuvants, mimetics and/or inhibitors of toxin-induced diarrhoea in any of a variety of drug screening techniques. The substance employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The abolition of substance activity or the formation of binding complexes between the substance and the agent being tested may be measured.

Another technique for screening provides for high throughput screening (HTS) of agents having suitable binding affinity to the substances and is based upon the method described in detail in WO 84/03564.

It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays.

PHARMACEUTICAL COMPOSITIONS

The present invention also provides a pharmaceutical composition comprising administering a therapeutically effective amount of the substance of the present invention and a pharmaceutically acceptable carrier, diluent or excipients (including combinations thereof).

The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be delivered using a a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestable solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be delivered by both routes.

Where the agent is to be delivered mucosally through the gastrointestinal mucosa, it should be able to remain stable during transit though the gastrointestinal tract; for example, it should be resistant to proteolytic degradation, stable at acid pH and resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

VACCINES

In one embodiment of the present invention, the substance is an adjuvant which is incorporated into a vaccine composition used to treat or prevent autoimmune disease, human T cell leukaemia, transplant rejection or graft-versus-host disease (GVHD), allergic or infectious disease.

In another embodiment of the invention, the vaccine composition may additionally comprise an antigen(s) or antigenic determinant(s). Suitable such antigens and/or antigenic determinants are disclosed in WO 99/34817.

Preferably the vaccine composition comprises an antigen and/or antigenic determinant.

Preferably the antigen is a self-antigen or a homologue thereof.

Preferably, the one or more substances of the present invention is used in the preparation of a therapeutic or prophylactic vaccine.

A "prophylactic vaccine" is a vaccine which is administered to naive individuals to prevent disease development.

A "therapeutic vaccine" is a vaccine which is administered to individuals with an existing infection to reduce or minimise the infection or to abrogate the immunopathological consequences of the disease.

The preparation of vaccines which contain one or more substances as an active ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents and pH buffering agents.

The vaccine composition may also comprise a combination of adjuvants which enhance the effectiveness of the vaccine. Examples of additional adjuvants which , in combination, may be effective include but are not limited to: aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionobacterium acnes*), *Bordetella pertussis*, polyribonucleotides, sodium alginate, lanolin, lysolecithin,

vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.) or Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Michigan).

Typically, adjuvants such as Amphigen (oil-in-water), Alhydrogel (aluminum hydroxide), or a mixture of Amphigen and Alhydrogel are used. Only aluminum hydroxide is approved for human use.

ADMINISTRATION

Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient. The dosages below are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited.

The compositions of the present invention may be administered by direct injection. The composition may be formulated for parenteral, mucosal, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Typically, each protein may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

The term "administered" includes delivery by viral or non-viral techniques. Viral delivery mechanisms include but are not limited to adenoviral vectors, adeno-associated viral (AAV) vectos, herpes viral vectors, retroviral vectors, lentiviral vectors, and baculoviral vectors. Non-viral delivery mechanisms include lipid mediated transfection, liposomes, immunoliposomes, lipofectin, cationic facial amphiphiles (CFAs) and combinations thereof. The routes for such delivery mechanisms include but are not limited to mucosal, nasal, oral, parenteral, gastrointestinal, topical, or sublingual routes.

The term "administered" includes but is not limited to delivery by a mucosal route, for example, as a nasal spray or aerosol for inhalation or as an ingestable solution; a parenteral route where delivery is by an injectable form, such as, for example, an intravenous, intramuscular or subcutaneous route.

The term "co-administered" means that the site and time of administration of each of the substance of the present invention and an additional entity such as an antigen and/or antigenic determinants are such that the necessary modulation of the immune system is achieved. Thus, whilst the substance and the antigen may be administered at the same moment in time and at the same site, there may be advantages in administering the substance at a different time and to a different site from the antigen. The substance and antigen may even be delivered in the same delivery vehicle - and the substance and the antigen may be coupled and/or uncoupled and/or genetically coupled and/or uncoupled.

The antigenic determinant and peptide or homologue or mimetic thereof may be administered separately or co-administered to the host subject as a single dose or in multiple doses.

The vaccine composition of the invention may be administered by a number of different routes such as injection (which includes parenteral, subcutaneous and intramuscular injection) intranasal, mucosal, oral, intra-vaginal, urethral or ocular administration.

The vaccines comprising the substance of the present invention are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, may be 1% to 2%. Oral formulations include such normally employed excipients as, for example,

pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer

DISEASES

The substance of the present invention is used to treat or prevent autoimmune disease, human T cell leukaemia, transplant rejection, allogeneic or xenogeneic transplant, graft-versus-host disease (GVHD), allergic or infectious diseases. Within the group "infectious diseases", are diseases in which, during infection, the infectious agent binds to, colonises or gains access across the mucosa are particularly preferred, as are diseases in which immunopathological mechanisms are commonly involved.

Examples of infectious diseases of the present invention include but are not limited to HSV-1, HSV-2, EBV, VZV, CMV, HHV-6, HHV-7 and HHV-8, hepatitis A, B, C, D and E, Neisseria meningitides, Haemophilus influenzae type B and Streptococcus pneumoniae, Legionella pneumophila and Mycobacterium tuberculosis, Neisseria gonnorheae, HIV-1, HIV-2 and Chlamydia trachomatism, E.coli, rotavirus, Salmonella enteritidis, Salmonella typhi, Helicobacter pylori, Bacillus cereus, Campylobacter jejuni and Vibrio cholerae, Staphylococcus aureus, Streptococcus pyogenes and Streptococcus mutans, malaria, Trypanasoma spp., Taxoplasma gondii, Leishmania donovani and Oncocerca spp.

Examples of allergic disorders of the present invention include but are not limited to diseases include asthma, allergic cough, allergic rhinitis and conjunctivitis, atopic eczema and dermatitis, urticaria, hives, insect bite allergy, dietary and certain drug allergies.

Examples of autoimmune diseases include but are not limited to diseases such as rheumatoid arthritis, multiple sclerosis and diabetes.

KITS

The present invention further provides diagnostic assays and kits comprising the substance of the present invention. Such kits may be used to prevent and/or treat and/or modulate the diseases of the present invention.

In one embodiment of the present invention, the kit may also comprise an antigen and/or antigenic determinant and/or a separate adjuvant for coadministration with said therapeutic or prophylactic composition.

Alternatively, a kit may be provided comprising mimetics of the present invention in the form of antibodies of the invention bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

SUMMARY

In summary, the present invention relates to a substance comprising any one or more of: an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB; but wherein the substance does not exhibit GM-1 binding activity.

The present invention also relates to an assay method for determing one or agents that are capable of interacting with and/or affecting the substance of the present invention wherein the assay comprises contacting the substance with an agent to be tested, and then determining whether or not the agent affects the substance.

Other aspects of the present invention are presented below by way of numbered paragraphs which include:

1. A peptide which comprises the sequence EVPGSQH, or a homologue or mimetic thereof.

2. A peptide according to paragraph 1, which comprises the sequence VEVPGSQHIDSQ.

3. A peptide according to paragraph 2 which comprises the sequence GATFQVEVPGSQHIDSQKKAI or the sequence GETFQVEVPGSQHIDSQKKAI.

4. A prophylactic or therapeutic composition which comprises a peptide according to any preceding paragraph or a homologue or mimetic thereof.

5. A prophylactic or therapeutic composition according to paragraph 4, which also comprises an antigen or an antigenic determinant.

6. A prophylactic or therapeutic composition according to paragraph 4 or 5, wherein the therapeutic or prophylactic agent is used as an adjuvant or immunomodulator.

7. A prophylactic or therapeutic composition according to paragraph 4 or 5, wherein the therapeutic or prophylactic agent is used to upregulate the production of antibodies at mucosal surfaces.

8. A prophylactic or therapeutic composition according to paragraph 4 or 5, wherein the therapeutic or prophylactic agent is used to prolong antigen presentation and give sustained immunological memory in a mammalian subject.

9. A prophylactic or therapeutic composition according to paragraph 4 or 5, wherein the therapeutic or prophylactic agent is used to downregulate the pathological components of Th1 and Th2-associated immune responses.

10. A prophylactic or therapeutic composition according to any of paragraph 4 to 9, which is used to treat or prevent autoimmune disease, human T cell leukaemia, transplant rejection, graft-versus-host disease or infectious diseases.

11. A prophylactic or therapeutic composition which comprises an agent which binds specifically to the ß4-α2 loop of EtxB or CtxB.

12. A prophylactic or therapeutic composition according to paragraph 11, wherein the agent is an antibody.

13. A prophylactic or therapeutic composition according to paragraph 11 or 12, which is used to treat diarrhoea.

14. A vaccine composition for use against a disease, comprising a peptide according to any of paragraphs 1 to 3 or a homologue or a mimetic thereof.

15. A vaccine composition according to paragraph 14 which also comprises an antigenic determinant.

16. A vaccine composition according to paragraph 14 or 15 which is used to treat or prevent infections diseases, autoimmune disease, human T cell leukaemia, transplant rejection or graft-versus-host disease (GVHD) diseases.

17. A kit comprising a therapeutic or prophylactic composition according to any of paragraphs 4 to 13.

The present invention will now be described only by way of example in which reference is made to the following Figures:

Figure 1 which shows a stereo ribbon drawing showing a B subunit of Etx/Ctx (from Sixma et al. J. Mol. Biol. (1993) 230:890-918, labels added);

Figure 2 which shows the identification of loop residues in CtxB involved in CD8+ T-cells apoptosis;

Figure 3 which shows mutant B subunits defective in CD8+ T-cell apoptosis retain ability to bind to cell surface receptors;

Figure 4 which shows total immunoglobulin levels against EtxB and EtxB (H57S) in sera from mice immunised intranasally with 10ug of each B-subunit;

Figure 5 which shows that His-57 in CtxB and EtxB defines a region necessary for adjuvanticity;

Figure 6 which shows E51-158 B subunit peptide exhibits an ability to induce immunomodulation of CD8+ T-cells.

EXAMPLES

Example 1

Identification of residues in the Glu-51 to Ile-58 loop of CtxB that trigger immunomodulatory effects on leukocytes

NIH male mice were sacrificed and mesenteric lymph node tissue was subsequently removed into Hanks balanced salt solution (HBSS without Calcium and Magnesium and supplemented with 20mM Hepes). Lymphocytes were then dispersed into the solution and away from fibrous tissue by gently pressing the tissue through a wire mesh. Following 3 washes in HBSS, the lymph node cells were resuspended in modified Eagle's medium (Gibco) (α-MEM) containing 20 mM Hepes, 4mM L-Glutamine, 100 IU/ml penicillin, 100ug/ml streptomycin, and $5x10^{-5}$ M2-

mercaptoethanol, at a concentration 2 x 10^6 cells/ml and then mixed without (PBS control) or with 3.45uM (40 ug/ml) of wild-type CtxB or wild-type EtxB, or various mutant B-subunits, namely EtxB(G33D), CtxB(E51A), CtxB(V52A), CtxB(P53A), CtxB(G54A), CtxB(S55A), CtxB(Q56A), CtxB(H57A), or CtxB(I58A) and incubated at 37°C for 96h. Thereafter, the cells were washed and resuspended in 0.4ml HBSS/20mM Hepes/0.1% sodium azide/10% rat serum. Phycoerythrin (PE) conjugated anti-CD8 (PharMingen) and FITC-conjugated anti-CD4 (PharMingen) were added at a dilution of 1/400 and the cells incubated on ice for a period of 30 min. Following antibody incubations, the cell suspensions were washed once in ISOTON (Becton-Dickinson) and resuspended in 0.4ml ISOTON. FACS analysis was carried out, with 10,000 events collected for each sample and then plotted using WinMDI software. The cells with FITC-bound anti-CD4 are depicted in the top-left hand quadrant of each figure; the cells with PE-bound anti-CD8 are depicted in the bottom right-hand quadrant, with the percentage number of events in each quadrant shown.

Results 1

The results in Figure 2 demonstrate, that incubation of MLN cells with wild-type CtxB or wild-type EtxB causes depletion of CD8+ T-cells; which does not occur if the cells are incubated in the presence of PBS (control) or a mutant form of EtxB, EtxB(G33D) which lacks an ability to bind to cell surface GM-1 ganglioside. An analysis of the CtxB mutants containing Ala substitutions in residues E51 to I58 revealed that CtxB(E51A) and CtxB(H57A) also failed to trigger CD8+ T-cell depletion. In addition, CtxB(V52A) and CtxB(I58A) exhibited a partial defect in triggering CD8+ T-cell depletion. These findings indicate that residues, E51 and H57 play an essential role in triggering modulatory effects on lymphocytes with a contributory role for residues V52 and I58.

Example 2

Mutant B-subunits defective in CD8+ T-cell apoptosis retain ability to bind to cell surface receptors

NIH male mice were sacrificed and mesenteric lymph node tissue was subsequently removed into Hanks balanced salt solution (HBSS without Calcium and Magnesium and supplemented with 20mM Hepes). Lymphocytes were then dispersed into the solution and away from fibrous tissue by gently pressing the tissue through a wire mesh. Following 3 washes in HBSS, the lymph cells were resuspended in 300 ml of pre-cooled and de-gassed MACS buffer (PBS, 5mM EDTA, 0.5% BSA, pH7.2). 50ml each of anti CD4 and anti-B220 MACS antibodies were added to the cells and the CD8 T-cell population purified by negative selection in a magnetic MACS column. The CD8+ T-cells containing population were washed and resuspended in modified Eagle's medium (Gibco) (α-MEM) containing 20 mM Hepes, 4mM L-Glutamine, 100 IU/ml penicillin, 100ug/ml streptomycin, and $5x10^{-5}$ M2-mercaptoethanol, at a concentration 2 x 10^6 cells/ml and then mixed without (PBS control) or with 3.45uM (40 ug/ml) of wild-type CtxB or wild-type EtxB, or various mutant B-subunits, namely EtxB(G33D), CtxB(E51A), CtxB(V52A), CtxB(P53A), CtxB(G54A), CtxB(S55A), CtxB(Q56A), CtxB(H57A), EtxB(H57S), or CtxB(I58A) and incubated on ice for 20 min. Thereafter, the cells were washed and resuspended in ice-cold 0.4ml HBSS/20mM Hepes/0.1% sodium azide/10% rat serum. Anti-EtxB monoclonal antibody 118-8 was added at a dilution of 1/500 to cells incubated EtxB, EtxB(G33D) or EtxB(H57S) and anti-CtxB monoclonal antibody LT-39 was added at a dilution of 1/800 to cells incubated with CtxB and CtxB mutants. After 30 min, the cells were washed and resuspended in HBSS/20mM Hepes/0.1% sodium azide/10% rat serum followed by addition of a FITC-labelled anti-mouse IgG antibody. Following incubation with the secondary antibody for 30 min, the cell suspensions were washed once in ISOTON (Becton-Dickinson) and resuspended in 0.4ml ISOTON. Levels of FITC fluorescence as a representative of the extent of binding of EtxB, CtxB and the various mutants to CD8+ T-cells was assessed by FACS analysis. The results of 10,000 events from each sample are plotted showing the fluorescence

intensity of CD8+ T-cells incubated in the absence of B-subunits (ie PBS contol; red line) versus the fluorescence attributable to bound B-subunits (black line) (Figure 3).

Results 2

The results in Figure 3 show that all B-subunits, except the non-binding mutant EtxB(G33D) bound to CD8+ T-cells to a similar extent. The fluorescence intensity detected after binding CtxB(H57A) and EtxB(H57S) was somewhat higher than that exhibited by wild-type B-subunits indicating that the two mutants have a greater avidity for the cell surface. This result is consistent with the finding that both CtxB(H57A) and EtxB(H57S) bind with a slightly higher avidity to GM-1-coated microtitre plates and exhibit a slightly higher Kd for GM-1 as determined by plasmon surface resonance (data not shown).

Example 3

Residue His-57 in EtxB is required to induce a potent anti-EtxB response

Groups of NIH female mice (n = 8) were immunised intranasally with 10ug EtxB or EtxB(H57S) in a volume of 20ul on 3 occasions at one week intervals. The mice were sacrificed and blood removed by cardiac puncture 14 days after the third immunisation. The sera were analysed for levels of anti-EtxB IgG antibodies by a GM-1-ELISA using microtitre plates coated with 1ug/ml EtxB. End point titres were determined (equivalent to a dilution giving an absorbance of 0.1 above background).

Results 3

The results in Figure 4 show that following intranasal immunisation with wild-type EtxB high titre serum anti-EtxB IgG antibody levels are induced (titre = 5757+/-785) whereas immunisation with EtxB(H57S) induces a significantly (p=0.001) lower response (titre 1205+/-222).

Example 4

Residue His-57 in EtxB and CtxB is necessary for the B-subunits to act as mucosal adjuvants

Groups of NIH female mice (n = 8) were immunised intranasally with 10ug ovalbumin (Ova) alone or with 10ug Ova mixed with either 10ug EtxB, CtxB, EtxB(H57S) or CtxB(H57A) and administered in a volume of 20ul on 3 occasions at one week intervals. In addition, two groups of mice were intranasally immunised with 10ug EtxB or CtxB as negative controls. All mice were sacrificed 14 days after the third immunisation and the blood removed by cardiac puncture. The sera were then analysed for levels of anti-Ova IgG antibodies by an ELISA using microtitre plates coated with 5ug/ml Ova. End point titres were determined (equivalent to a dilution giving an absorbance of 0.1 above background).

Results 4

The results in Figure 5 show that both wild-type EtxB and CtxB act as mucosal adjuvants, substantially augmenting the anti-Ova response compared with mice immunised with Ova alone (compare lanes 4 and 5 with lane 1). By contrast, when Ova was admixed with EtxB(H57S) (lane 6) or CtxB(H57A) (lane 7) the anti-Ova response induced was substantially less than that triggered by inclusion of the wild-type B-subunits. The data demonstrate that CtxB(H57A) lacks adjuvant activity. This confirms the importance of the B-subunit E51-I58 loop, and in particular H57 in mediating the immunomodulatory properties of the molecule.

Example 5

A synthetic peptide EVPGSQHI corresponding to the E51 to I58 loop of EtxB and CtxB possesses immunomodulatory properties

To assess whether a synthetic peptide corresponding to the E51 to I58 loop of EtxB (and CtxB) causes depletion of CD8+ T-cells from MLN cultures, mesenteric lymph node cells were isolated as described in Example 1, and incubated with various concentrations (0.1uM - 20uM) of the EVPGSQHI peptide or a randomly selected control peptide, LRNETTTTKGDYC. After 96h incubation at 37°C the cells were washed and resuspended in 0.4ml HBSS/20mM Hepes/0.1% sodium azide/10% rat serum and then assessed for the relative proportion of CD4+ and CD8+ cells by FACS analysis, in an identical fashion to that reported in Example 1. The percentages of CD8+ T-cells remaining in cultures treated with the EVPGSQHI peptide (closed red circles and line) or the control peptide (closed black squares and line) was determined and was plotted graphically against concentration of peptide used (Figure 6).

Results 5

The results in Figure 6 show that incubation of MLN cultures in the presence of the EVPGSQHI peptide causes a reduction in CD8+ T-cell numbers, in contrast to treatment with a control peptide. This shows that a synthetic peptide corresponding to the E51 to I58 loop of EtxB and CtxB is active in exerting direct modulatory effects on lymphocytes.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A substance comprising any one or more of: an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB; but wherein the substance does not exhibit GM-1 binding activity.

2. A substance as defined in claim 1 for use in medicine.

3. A substance as defined in claim 1 for use as an immunomodulator.

4. A substance as defined in claim 1 for use as an adjuvant.

5. A substance as defined in claim 1 for use as an inhibitor for toxin-induced diarrhoea.

6. A substance according to any one of the preceding claims wherein the substance additionally comprises an antigen or an antigenic determinant.

7. A pharmaceutical composition comprising the substance according to any one of claims 1 to 6, optionally admixed with one or more pharmaceutically acceptable carrier(s), diluent(s) or excipient(s).

8. Use of a substance as defined in any one of claims 1 to 7 for use in the manufacture of a medicament that is capable of treating and/or preventing and/or modulating a disease and/or condition associated with an immune disorder and/or a toxin induced diarrhoea disease.

9. An assay method for determining one or more agents that are capable of interacting with and/or affecting the substance according to any one of claims 1 to 7; wherein the assay comprises contacting the substance with an agent to be tested, and then determining whether or not the agent affects the substance.

10. An agent identified by the assay method according to claim 9.

11. A method of treatment, comprising administering to a subject in need of treatment of and/or prevention of and/or modulation of a disease and/or condition associated with an immune disorder and/or a toxin mediated disorder a substance as defined in any one of claims 1 to 7.

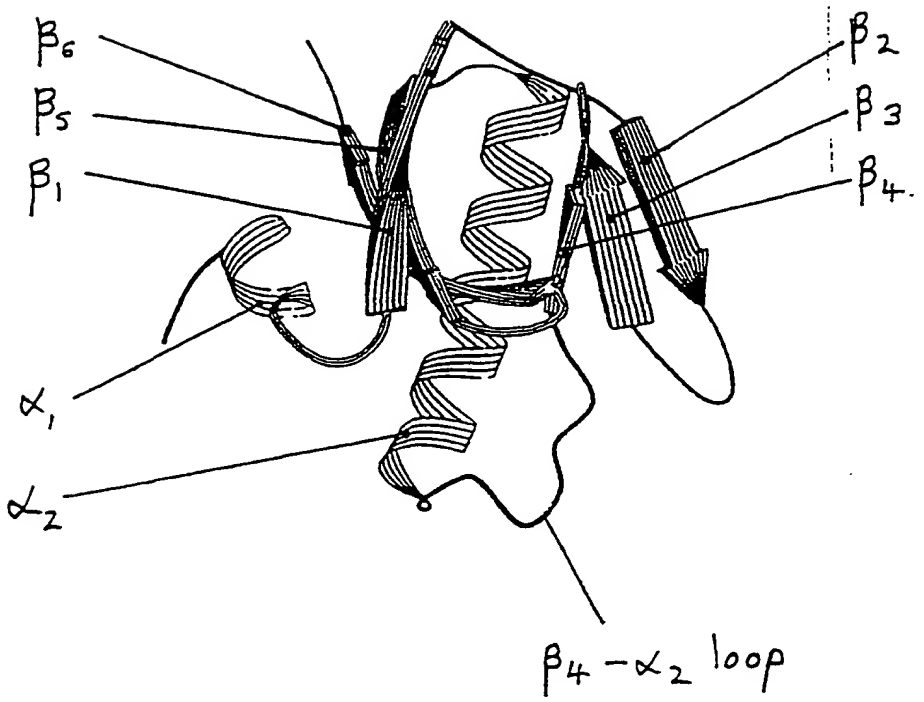


Figure 1

Identification of loop residues in CtxB involved in CD8+ T-cells apoptosis

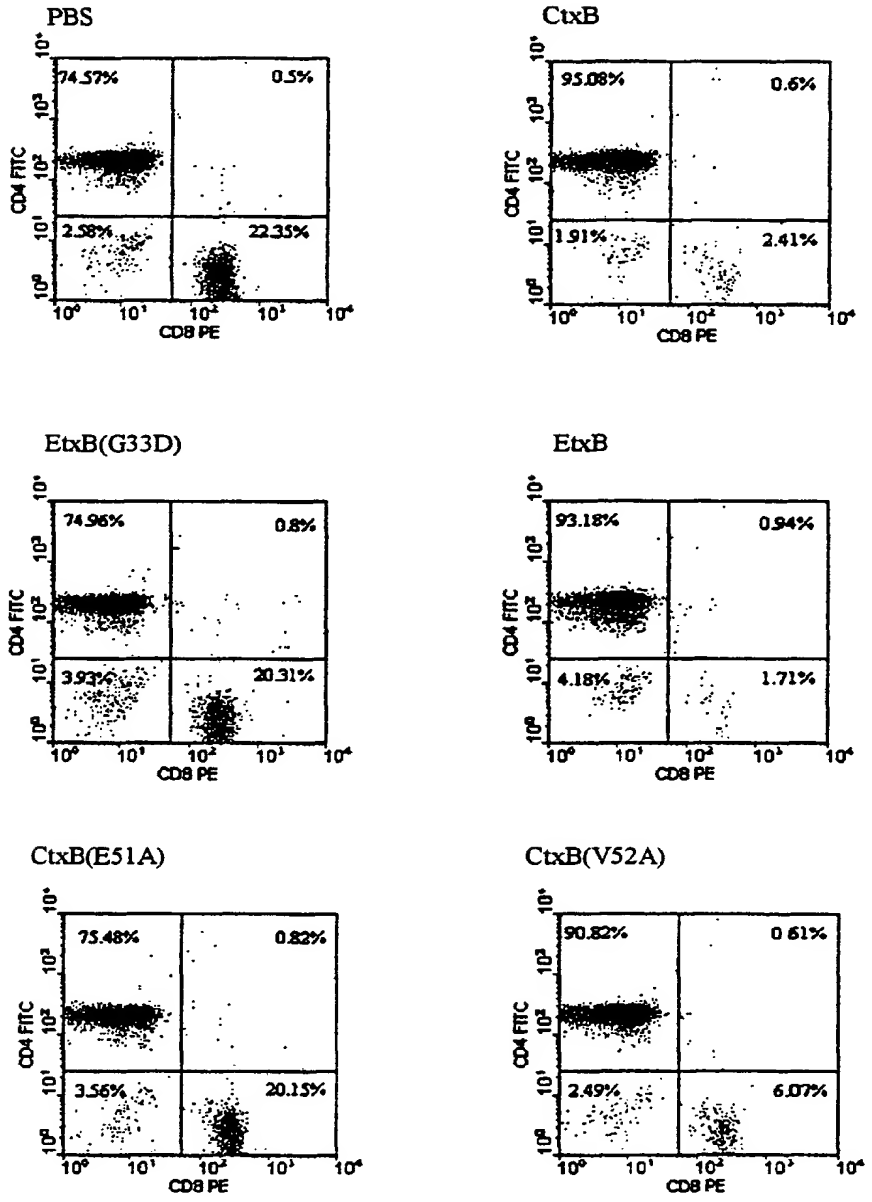
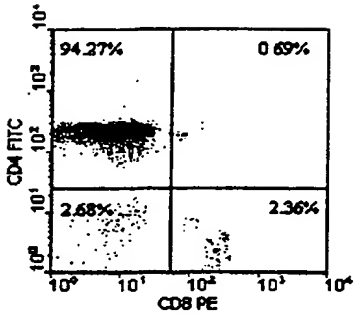
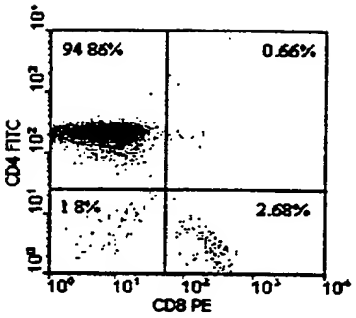


Figure 2

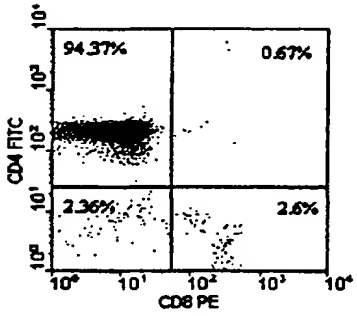
CtxB(P53A)



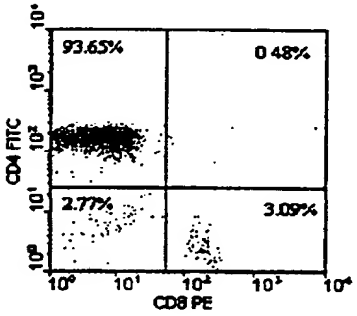
CtxB(G54A)



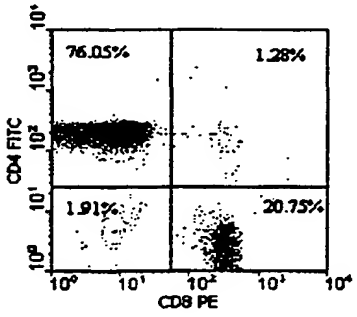
CtxB(S55A)



CtxB(Q56A)



CtxB(H57A)



CtxB(I58A)

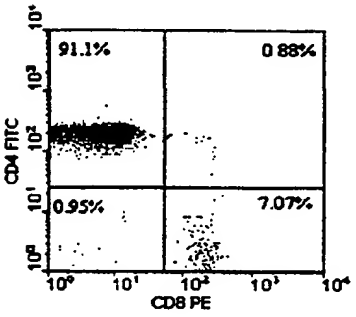


Figure 2 contd....

Mutant B subunits defective in CD8+ T-cell apoptosis retain ability to bind to cell surface receptors

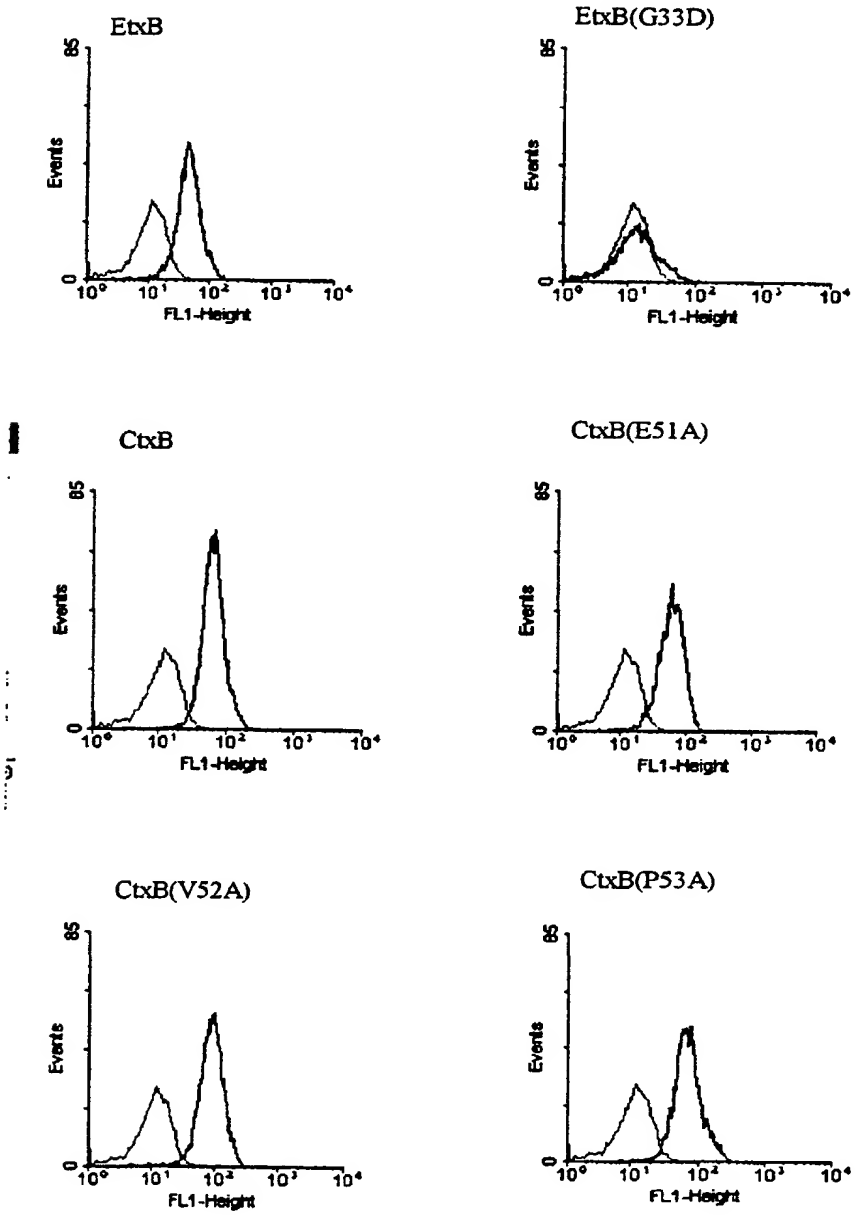


Figure 3

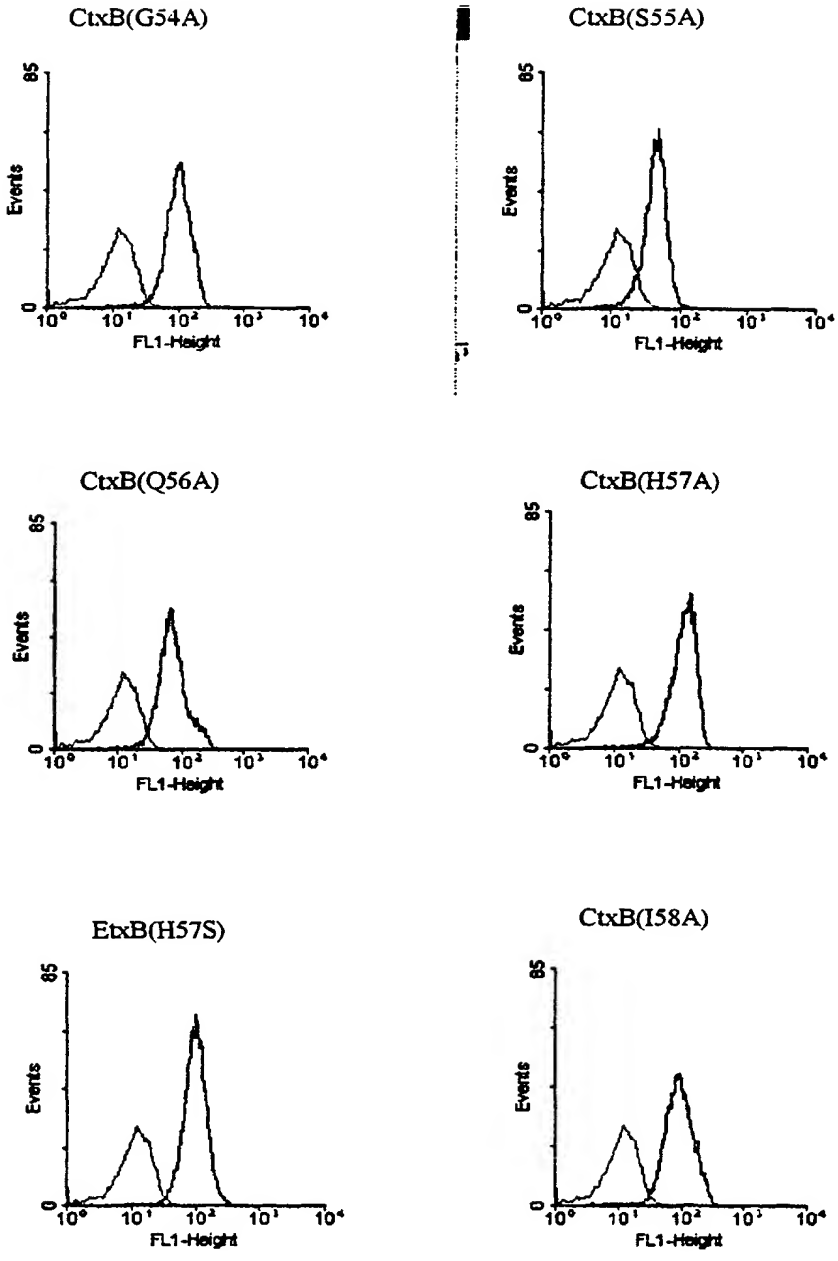


Figure 3 contd....

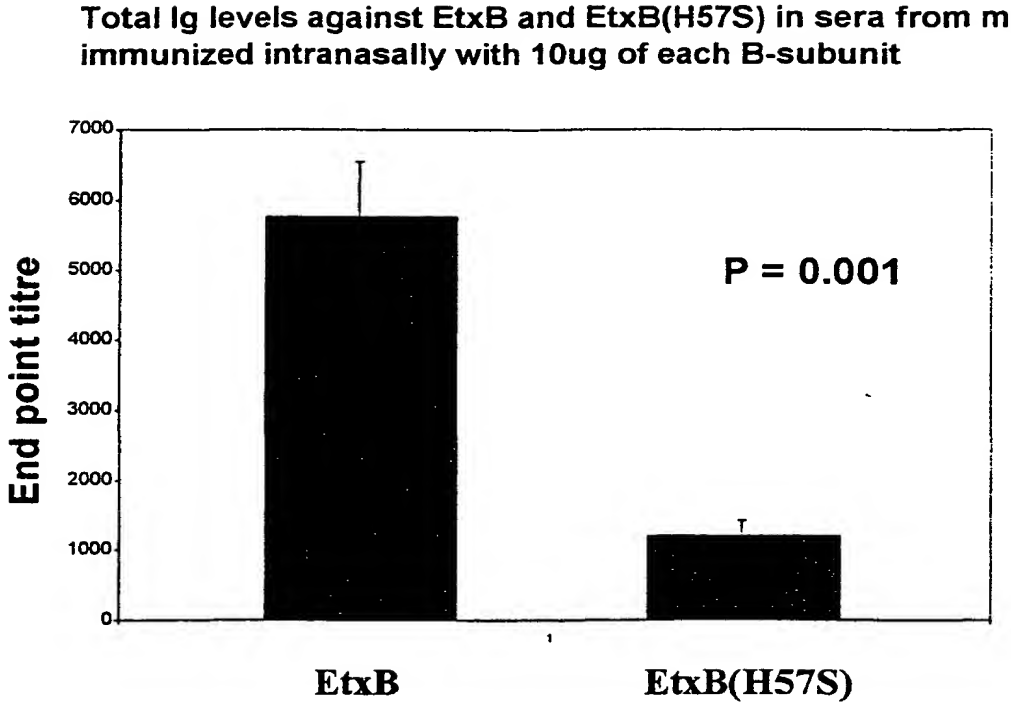

Total Ig levels against EtxB and EtxB(H57S) in sera from mice immunized intranasally with 10ug of each B-subunit
End point titre
7000
6000
5000
4000
3000
2000
1000
0
P = 0.001
EtxB
EtxB(H57S)

Figure 4

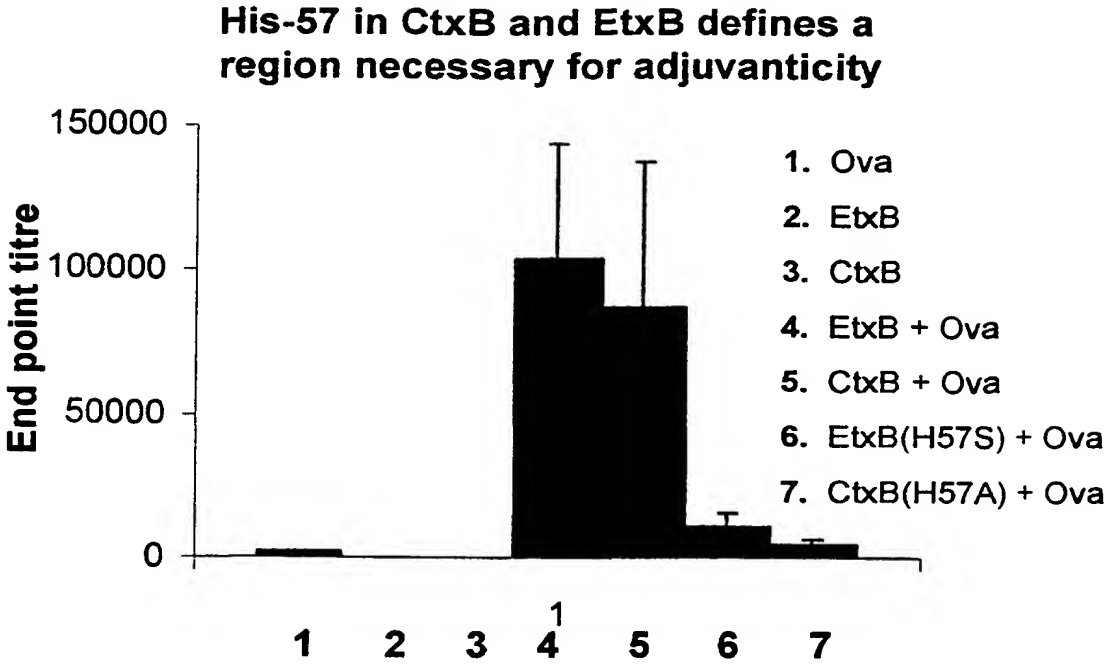

His-57 in CtxB and EtxB defines a region necessary for adjuvanticity
End point titre
150000
100000
50000
0
1. Ova
2. EtxB
3. CtxB
4. EtxB + Ova
5. CtxB + Ova
6. EtxB(H57S) + Ova
7. CtxB(H57A) + Ova
1 2 3 4 5 6 7

Figure 5

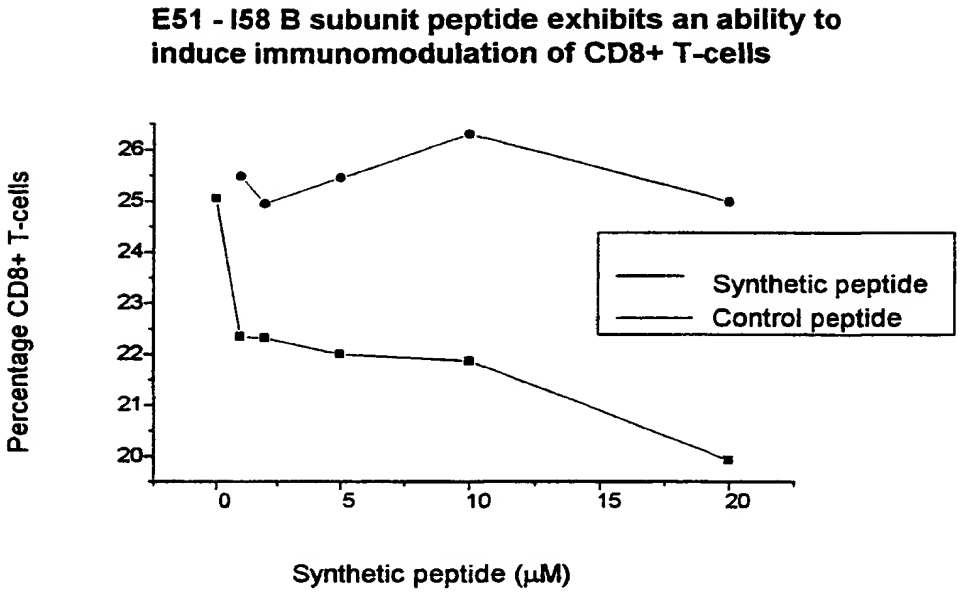

E51 - I58 B subunit peptide exhibits an ability to induce immunomodulation of CD8+ T-cells
Percentage CD8+ T-cells
26
25
24
23
22
21
20
0
5
10
15
20
Synthetic peptide (μM)
Synthetic peptide
Control peptide

Figure 6

SEQUENCE LISTING

SEQ ID No 1

GAA GTA CCA GGT AGT CAA CAT ATA GAT

SEQ ID No 2

EVPGSQH

SEQ ID No 3

VEVPGSQHIDSQ

SEQ ID No 4

GATFQVEVPGSQHIDSQKKAI

SEQ ID No 5

GETFQVEVPGSQHIDSQKKAI

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02970

A CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/28 C07K14/245 A61P37/02 A61K39/108 A61K39/112
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 95 29701 A (YEDA RES & DEV ;MIRELMAN DAVID (IL); MARKS ROBERT S (IL); SELA MIC) 9 November 1995 (1995-11-09) claim 8	1,2
X	WO 95 20657 A (GX BIOSYSTEMS AS ;SOKURENKO EVGENI VENIAMINOVIC (US); HASTY DAVID) 3 August 1995 (1995-08-03) page 58, line 9	1
X	EP 0 095 426 A (CENTRE NAT RECH SCIENT ;PASTEUR INSTITUT (FR)) 30 November 1983 (1983-11-30) claim 7	1-3
A	DE 34 30 894 A (YEDA RES & DEV) 14 March 1985 (1985-03-14) claims; examples	1

-/--

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 February 2000	15/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02970

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 90 03437 A (UNIV LIEGE) 5 April 1990 (1990-04-05) claims; figure 5; examples	1-8,11
A	WO 96 06627 A (UNIV TULANE) 7 March 1996 (1996-03-07) page 13, line 3 -page 14, line 22; claims	1-8,11
X	WO 85 02611 A (SCRIPPS CLINIC RES) 20 June 1985 (1985-06-20) claim 8	1-8,11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/02970

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9529701 A	09-11-1995	IL 109519 A	12-03-1999
		CA 2189469 A	09-11-1995
		EP 0759779 A	05-03-1997
WO 9520657 A	03-08-1995	AU 1532795 A	15-08-1995
		CA 2180726 A	03-08-1995
		EP 0738325 A	23-10-1996
EP 0095426 A	30-11-1983	FR 2527445 A	02-12-1983
DE 3430894 A	14-03-1985	IL 69558 A	30-06-1988
		FR 2550943 A	01-03-1985
		GB 2145419 A,B	27-03-1985
		US 4751064 A	14-06-1988
WO 9003437 A	05-04-1990	FR 2636842 A	30-03-1990
		AT 123525 T	15-06-1995
		DE 68923006 D	13-07-1995
		EP 0445128 A	11-09-1991
WO 9606627 A	07-03-1996	AU 709779 B	09-09-1999
		AU 3233795 A	22-03-1996
		BR 9508633 A	30-09-1997
		CA 2198586 A	07-03-1996
		CZ 9700562 A	15-10-1997
		EP 0777490 A	11-06-1997
		FI 970799 A	24-04-1997
		HU 77869 A	28-09-1998
		JP 10505059 T	19-05-1998
		NO 970993 A	25-04-1997
		NZ 291262 A	25-02-1999
		PL 318930 A	21-07-1997
		ZA 9506412 A	11-03-1996
WO 8502611 A	20-06-1985	US 4603049 A	29-07-1986
		AT 39700 T	15-01-1989
		AU 572821 B	19-05-1988
		AU 2436884 A	02-08-1984
		AU 3747185 A	26-06-1985
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		DK 421584 A	03-09-1984
		EP 0117367 A	05-09-1984
		EP 0165307 A	27-12-1985
		ES 528649 A	01-05-1985
		FI 843451 A	03-09-1984
		FI 853082 A	12-08-1985
		GR 79805 A	31-10-1984
		JP 61500664 T	10-04-1986
		NO 843464 A	31-08-1984
		NO 853153 A	13-09-1985
		PH 23161 A	19-05-1989
		PT 77910 A,B	01-01-1984
		US 4758655 A	19-07-1988
		WO 8402700 A	19-07-1984
		US 4886663 A	12-12-1989
		ZA 8309512 A	29-08-1984

PATENT COOPERATION TREATY

REC'D 08 DEC 2000
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P007438WOCTH		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/02970	07/09/1999	07/09/1998
International Patent Classification (IPC) or national classification and IPC C07K14/28		
Applicant UNIVERSITY OF BRISTOL et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 13 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
28/03/2000	05.12.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Mundel, C Telephone No. +49 89 2399 7314

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/02970

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-45 as originally filed

Claims, No.:

1-11 as originally filed

Drawings, sheets:

1/8-8/8 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 10.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 10 are so unclear that no meaningful opinion could be formed (*specify*):
see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion

could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-9 and 11
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-9 and 11
Industrial applicability (IA)	Yes:	Claims	1-9
	No:	Claims	11 (See Citations and explanations)

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I

Basis of the opinion

A sequence listing has been filed with the present application on the date of 11.10.99. This listing contains SEQ ID NO:1 to 5 (page 1).

Re Item II

Priority

The priority document of the present application was not available at the time where this preliminary opinion has been drafted. The present analysis is based on the hypothesis that all the claims have a priority right corresponding to the date of filing of the priority document 07.09.98.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claim 10 lacks clarity and is not supported by the description (see points VIII-5 and VIII-6). Therefore, a meaningful evaluation regarding novelty, inventive step, and industrial applicability can not be carried out.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. **Reference is made to the following documents :**

D1: WO 95 29701 A (YEDA RES & DEV ;MIRELMAN DAVID (IL); MARKS ROBERT S (IL); SELA MIC) 9 November 1995 (1995-11-09)

D2: WO 95 20657 A (GX BIOSYSTEMS AS ;SOKURENKO EVGENI VENIAMINOVIC (US); HASTY DAVID) 3 August 1995 (1995-08-03)

D3: EP-A-0 095 426 (CENTRE NAT RECH SCIENT ;PASTEUR INSTITUT (FR)) 30 November 1983 (1983-11-30)

D4: DE 34 30 894 A (YEDA RES & DEV) 14 March 1985 (1985-03-14)

D5: WO 85 02611 A (SCRIPPS CLINIC RES) 20 June 1985 (1985-06-20)

2. The present application discloses the identification of an amino acid sequence which is important for the role of the CtxB toxin in triggering the depletion of CD8+ T-cells, in inducing a potent anti-EtxB response and to act as mucosal adjuvant However, the claims are directed to a substance comprising an amino acid comprising the sequence EVPGSQHI for use in medicine and more particularly as immunomodulator, adjuvant or inhibitor for toxin-induced diarrhoea. The claims also refer to a pharmaceutical composition comprising said substance, to the use of said substance in the manufacture of medicament, to a method for determining agents capable of interacting with "or affecting" said substance and to an agent identified by said method, and to a method of treatment comprising administering said substance to a subject.

3. **Lack of novelty and inventive step; articles 33(2) and 33(3) PCT.**

3.1 The document D1 discloses conjugates of an antigen selected from the group of a toxin or a fragment thereof, a toxoid and/or an adherence antigen derived from an infecting agent covalently bound to an inert carrier (Abstract, lines 1-3). Said conjugates are for use in **vaccines** for oral immunization against infecting agents (Abstract, lines 3-4). One of the antigen disclosed in D1 is the **peptide CTP3** consisting of the amino acid 50-64 of the cholera toxin B subunit chain. This peptide **comprises** the sequence EVPGSQHI (= SEQ ID NO:2) (p. 3, lines 14-19). D1 also states that a fragments from toxin from other enteric pathogens like Escherichia coli-LT (=EtxB) can be used. An experiment shows that the colostrum of female rabbits immunized with the conjugate silica-cholera toxin B subunit derived synthetic peptide CTP3 contains IgA directed against the native cholera toxin (p. 12, lines 9-17). A ELISA test in order to detect antibodies raised against two conjugates : Si-TGB-CTP3 and TGB-CTP3 - which both comprise the peptide CTP3 **linked to the thyroglobulin** - is also disclosed (p. 22, lines 10-23).

A solution containing the CTP3 peptide is considered as fulfilling the definition of claim 1 for the following reasons :

- Due to its small size (15 amino acids) and due to its localization in the

sequence of the cholera toxin B subunit sequence (AAs 50-64), the polypeptide CTP3 obviously has no GM1-binding activity.

- Antibodies directed against the CTP3 peptide can also recognize the native cholera toxin. Due to the lack of clarity mentioned in point VIII-1 and more especially point VIII-1 (v), the CTP3 peptide can be considered as being "capable of acting in a manner that is the same or is similar to CtxB" at least as far as the antigenicity is concerned.
- Since the CTP3 peptide contains the sequence shown in SEQ ID NO:2 which, according to the present application, plays an essential role in triggering the depletion of CD8+ T-cells (Example 1, p. 41-42), is required to induce a potent anti-EtxB response (Example 3, p.43) and is necessary for the toxin B-subunit to act as mucosal adjuvant (Example 4, p. 44), said peptide will, **per se**, have all those activities.

Thus, claims 1 and 7 can not be considered as new or inventive in the sense of articles 33(2) and 33(3) PCT.

Moreover, since the use of the CTP3 peptide as a vaccine has already been disclosed, the subject-matter of claims 2-5 (which are considered as first medical use by the European Patent Office) and 11 can not be considered as new or inventive in the sense of articles 33(2) and 33(3) PCT.

Furthermore, the thyroglobulin which is linked to the CTP3 peptide in the conjugates disclosed in D1 is considered as an additional antigen. Thus, the subject-matter of claim 6 can not be considered as new or inventive (articles 33(2) and 33(3) PCT).

Claim 8 of the present application is considered as a second medical use claim by the European Patent Office. Due to the clarity problem mentioned in point VIII-4, the vaccine against the cholera toxin containing the CTP3 peptide disclosed in D1 fit the definition of a medicament according to claim 8. Thus, claim 8 can not be considered as novel or inventive (articles 33(2) and 33(3) PCT).

The ELISA test used to determine if IgA (=agent) will bind to the conjugates comprising the CTP3 peptide (=substance according to any one of claims 1

to 7) fit the definition of the assay method disclosed in claim 9. Thus, the subject-matter of claim 9 can not be considered as new or inventive in the sense of article 33(2) and 33(3) PCT (see also point VIII-5).

3.2 The documents D2-D5 are also considered as relevant for the evaluation of the novelty and inventiveness of the claims and will be discussed briefly :

D2 refers, inter alia, to fusion proteins containing a neutralizing epitope of the cholera toxin B chain (which is the same as the CTP3 peptide of D1) inserted in two different positions in the FimH adhesin of type 1 fimbriae. The binding of anti-CtxB antibodies to the fusion protein has been tested. This document deprives claims 1, 6, 7 and 9 of novelty and inventiveness for the same reasons as those disclosed in point V-3.1 above.

D3 refers to new medicaments comprising at least one sequence of the cholera toxin B subunit, inter alia the sequence 50-75 which comprises the sequence EVPGSQHI. Said sequence has been used for the manufacture of a vaccine against cholera and other human and animal infections due to Escherichia coli enterotoxin LT (=EtxB). The binding of anti-CtxB antibodies to the fragment 50-75 has also been tested. For the reasons mentioned in point 3.1 above, claims 1-9 and 11 can not be considered as new or inventive over the teaching of D3.

D4 discloses, inter alia, the use of the CTP3 peptide as a vaccine against cholera and heat-labile E.coli toxin. The teaching of this document is almost the same as document D1. Thus claims 1-9 and 11 can not be considered as novel or inventive over the teaching of D4.

D5 discloses the use of peptides containing 10 to 35 amino acids residues corresponding the amino acids 35 to 95 from the B-subunit of the heat-labile enterotoxin of Escherichia coli - some of which contain the sequence EVPGSQHI (p. 75-77) - in polymers as the active ingredient of a vaccine for protection against infection by heat-labile enterotoxin-producing bacteria. The use of said peptides coupled to carriers is also disclosed. Therefore, claims 1-9 and 11 can not be considered to be novel or inventive over the teaching of D5 (see point 3.1 for explanations).

4. Industrial applicability; article 33(4) PCT.

Claim 11 refers to a method of treatment of the human or animal body.
For the assessment of the present claim 11 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII
Certain observations on the international application

Lack of clarity; article 6 PCT.

1. Claim 1 of the present application lacks clarity for the following reasons :

   (i) The use of the vague term "substance" renders the scope of the claim unclear since it can include lots of different components in addition to the amino acid sequence comprising the sequence shown in SEQ ID NO:2.

   (ii) Claim 1 refers to a "variant", "homologue", "derivative" or "mimetic" of an amino acid sequence. The use of these terms renders the scope of the claim unclear since there is no clear definition of what such a variant, homologue,

derivative or mimetic should be.
Moreover, it is not clear if these terms apply to the amino acid sequence shown in SEQ ID NO:2 or to the amino acid sequence comprising said sequence. In this written opinion, it has been assumed that said terms refer to the amino acid sequence shown in SEQ ID NO:2 which represents the only technical feature of claim 1.
Therefore, the IPEA considers that a protein comprising an amino acid sequence differing from the sequence disclosed in SEQ ID NO:2 by one or even more amino acids can still be considered as **comprising** a "variant", "homologue" or "derivative" of said sequence. Some well-known proteins probably fit this definition.

(iii) It is not clear what is meant by "fragment" of an amino acid sequence of 8 amino acids. The attention of the applicant is drawn to the fact that even a single amino acid could be reasonably considered as a fragment of a 8 amino acid sequence. Thus, every protein can be considered as comprising a fragment of SEQ ID NO:2 and lots of well-known proteins contain at least 2 or 3 consecutive amino acids of SEQ ID NO:2.

(iv) Due to the clarity problems mentioned in points 1 (i), (ii) and (iii) above, the only distinction between the substance of the present application and well-known proteins - like, for example, the fragments of the EtxB and CtxB toxins amino acid sequences disclosed in D1-D5 - is the fact that the substance of claim 1 is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB, but does not exhibit GM-1 binding activity, i.e. **by the result to be achieved.**
According to the PCT Gazette of the 29.10.98 "PCT International Preliminary Examination Guidelines", Chapter III-4.7 : "The area defined by the claims must be as precise as the invention allows. As a general rule, claims which attempt to define the invention, or a feature thereof, by a result to be achieved should be objected to".
The substance of claim 1 should be described in terms of the technical features - for example specific amino acid sequences - which cause the substance of claim 1 to be capable of acting in a manner that is the same as or similar to EtxB and/or CtxB without exhibiting GM-1 binding activity.

(v) Claim 1 refers to a substance which is "capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB". This wording renders the scope of the claim unclear since there is no clear definition in the present application, and especially in the claims, of which activities of EtxB and CtxB are meant.
Moreover, the addition of the wording "capable of acting in a manner that is the same as **or is similar to** EtxB and/or CtxB" renders the scope of the claim even more unclear.

(vi) Moreover, the attention of the applicant is drawn to the fact that, in the present application, there is **no example** of a substance fulfilling the requirements of claim 1 since the **only** substance comprising an amino acid sequence **comprising** the sequence disclosed in SEQ ID NO:2 and which **does not exhibit** a GM-1 binding activity is the EtxB(G33D) mutant which **does not cause** a depletion of $CD8^+$ T-cells (Example 1) and which **does not**, therefore, act "in a manner that is the same as or is similar to EtxB". Thus, there is no support in the present application that a substance comprising an amino acid sequence comprising the sequence disclosed in SEQ ID NO:2 and which **does not have** a GM-1 binding activity **could retain** the ability to act "in a manner that is the same as or is similar to EtxB and/or CtxB" (article 5 PCT).

2. As a general remark about the different uses of the substance of claim 1, the attention of the applicant is drawn to the fact that, since there is no example of a substance fulfilling the definition of claim 1 in the present application, there are also no evidences that such a substance could be used as an immunomodulator, an adjuvant, an inhibitor of toxin-induced diarrhoea or could be used in the manufacture of a medicament for the treatment and/or the prevention and/or the modulation of a disease and/or condition associated with an immune disorder and/or a toxin induced diarrhoea disease. Thus, the different uses claimed for the substance of the present application are not considered as being supported by the description (article 5 PCT).

3. Claim 3 refers to the use of the substance of claim 1 as an immunomodulator. The wording of this claim is unclear since there is no clear definition of what is meant

by "**immunomodulator**". In the broad sense of this term, each antigen can be considered as being an immunomodulator since it promotes the expansion of the pool of T and B-cells recognizing this specific antigen.

4. Claim 8 is unclear for the following reasons :

   (i) The attention of the applicant is drawn to the fact that the term "use" is redundant : use ... for use in the manufacture.

   (ii) There is no clear definition of what "modulating a disease" should be.

   (iii) There is no clear definition of what is meant by an "immune disorder".

   (iv) It is not clear if the wording "associated with an immune disorder" refers only to the term "condition" or also to the term "disease".

5. Claim 9 refers to an assay method for determining agents that are capable of interacting with and/or affecting the substance according to any of claims 1 to 7. The wording of said claim is unclear for the following reasons :

   (i) There is no clear definition of what "affecting the substance" should mean.

   (ii) Since the substance is not limited to an amino acid sequence comprising the sequence shown in SEQ ID NO:2 but can also include almost any other compounds (see point VIII-1 (i)), the assay method of claim 9 will not be limited to determine compounds interacting with or "affecting" the amino acid sequence comprising the sequence shown in SEQ ID NO:2 but will also include the detection of **any change in any of the compounds** included in the substance of claim 1. Lots of the methods encompassed by the present wording of claim 9 are well-known and will deprive claim 9 of novelty.

6. Claim 10 refers to an agent identified by the assay method according to claim 9. Due to the clarity problem mentioned in point VIII-5 above, the IPEA considers that most of the compounds encompassed by said claim will be well-known compounds.

Even if claim 9 should be restricted to the detection of compounds interacting with and/or affecting the amino acid sequence comprised in the substance of claim 1, the IPEA considers that claim 10 would still be unclear since the agents of said claim are **not** characterized by any **technical features**. Moreover, there is no description in the present application of what such an agent should be, thus the IPEA considers that the agents claimed are not supported by the description of the present application (article 5 PCT).

7. Claim 11 is unclear for the following reasons :

(i) There is no clear definition of what a "condition associated with an immune disorder and/or a toxin mediated disorder" should be.

(ii) It is not clear what is meant by "**modulation** of a disease and/or condition associated with an immune disorder and/or a toxin mediated disorder".

ENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P/7438.WOCTH		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 02970	07/09/1999	07/09/1998
Applicant		
UNIVERSITY OF BRISTOL et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

PEPTIDE FRAGMENTS OF CHOLERA TOXIN B OR ENTEROTOXIN B AS VACCINE ADJUVANTS

5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02970

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/28 C07K14/245 A61P37/02 A61K39/108 A61K39/112
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 29701 A (YEDA RES & DEV ;MIRELMAN DAVID (IL); MARKS ROBERT S (IL); SELA MIC) 9 November 1995 (1995-11-09) claim 8	1,2
X	WO 95 20657 A (GX BIOSYSTEMS AS ;SOKURENKO EVGENI VENIAMINOVIC (US); HASTY DAVID) 3 August 1995 (1995-08-03) page 58, line 9	1
X	EP 0 095 426 A (CENTRE NAT RECH SCIENT ;PASTEUR INSTITUT (FR)) 30 November 1983 (1983-11-30) claim 7	1-3
A	DE 34 30 894 A (YEDA RES & DEV) 14 March 1985 (1985-03-14) claims; examples	1

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 February 2000	15/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Fuhr, C

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]B 99/02970

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 03437 A (UNIV LIEGE) 5 April 1990 (1990-04-05) claims; figure 5; examples	1-8,11
A	WO 96 06627 A (UNIV TULANE) 7 March 1996 (1996-03-07) page 13, line 3 -page 14, line 22; claims	1-8,11
X	WO 85 02611 A (SCRIPPS CLINIC RES) 20 June 1985 (1985-06-20) claim 8	1-8,11

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/02970

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9529701	A	09-11-1995	IL	109519 A	12-03-1999
			CA	2189469 A	09-11-1995
			EP	0759779 A	05-03-1997
WO 9520657	A	03-08-1995	AU	1532795 A	15-08-1995
			CA	2180726 A	03-08-1995
			EP	0738325 A	23-10-1996
EP 0095426	A	30-11-1983	FR	2527445 A	02-12-1983
DE 3430894	A	14-03-1985	IL	69558 A	30-06-1988
			FR	2550943 A	01-03-1985
			GB	2145419 A,B	27-03-1985
			US	4751064 A	14-06-1988
WO 9003437	A	05-04-1990	FR	2636842 A	30-03-1990
			AT	123525 T	15-06-1995
			DE	68923006 D	13-07-1995
			EP	0445128 A	11-09-1991
WO 9606627	A	07-03-1996	AU	709779 B	09-09-1999
			AU	3233795 A	22-03-1996
			BR	9508633 A	30-09-1997
			CA	2198586 A	07-03-1996
			CZ	9700562 A	15-10-1997
			EP	0777490 A	11-06-1997
			FI	970799 A	24-04-1997
			HU	77869 A	28-09-1998
			JP	10505059 T	19-05-1998
			NO	970993 A	25-04-1997
			NZ	291262 A	25-02-1999
			PL	318930 A	21-07-1997
			ZA	9506412 A	11-03-1996
WO 8502611	A	20-06-1985	US	4603049 A	29-07-1986
			AT	39700 T	15-01-1989
			AU	572821 B	19-05-1988
			AU	2436884 A	02-08-1984
			AU	3747185 A	26-06-1985
			DK	364685 A	09-08-1985
			DK	421584 A	03-09-1984
			EP	0117367 A	05-09-1984
			EP	0165307 A	27-12-1985
			ES	528649 A	01-05-1985
			FI	843451 A	03-09-1984
			FI	853082 A	12-08-1985
			GR	79805 A	31-10-1984
			JP	61500664 T	10-04-1986
			NO	843464 A	31-08-1984
			NO	853153 A	13-09-1985
			PH	23161 A	19-05-1989
			PT	77910 A,B	01-01-1984
			US	4758655 A	19-07-1988
			WO	8402700 A	19-07-1984
			US	4886663 A	12-12-1989
			ZA	8309512 A	29-08-1984

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
D. YOUNG & CO.
Attn. HARDING, C.
21 New Fetter Lane
London EC4A 1DA
UNITED KINGDOM

DARH-15-4-00

CTH

Records noted

Applicant's or agent's file reference P/7438.WOCTH	Date of mailing (day/month/year) 15/02/2000
International application No. PCT/GB 99/02970	FOR FURTHER ACTION See paragraphs 1 and 4 below
Applicant UNIVERSITY OF BRISTOL et al.	International filing date (day/month/year) 07/09/1999

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the International application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nina Vercio

Form PCT/ISA/220 (July 1998)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P/7438.WOCTH		
International application No.	International filing date (day/month/year)	(Earliest) Priority Date (day/month/year)
PCT/GB 99/02970	07/09/1999	07/09/1998
Applicant		
UNIVERSITY OF BRISTOL et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readable form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] Certain claims were found unsearchable (See Box I).

3. [ ] Unity of invention is lacking (see Box II).

4. With regard to the title,

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

PEPTIDE FRAGMENTS OF CHOLERA TOXIN B OR ENTEROTOXIN B AS VACCINE ADJUVANTS

5. With regard to the abstract,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]GB 99/02970

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/28 C07K14/245 A61P37/02 A61K39/108 A61K39/112
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 29701 A (YEDA RES & DEV ;MIRELMAN DAVID (IL); MARKS ROBERT S (IL); SELA MIC) 9 November 1995 (1995-11-09) claim 8	1,2
X	WO 95 20657 A (GX BIOSYSTEMS AS ;SOKURENKO EVGENI VENIAMINOVIC (US); HASTY DAVID) 3 August 1995 (1995-08-03) page 58, line 9	1
X	EP 0 095 426 A (CENTRE NAT RECH SCIENT ;PASTEUR INSTITUT (FR)) 30 November 1983 (1983-11-30) claim 7	1-3
A	DE 34 30 894 A (YEDA RES & DEV) 14 March 1985 (1985-03-14) claims; examples	1
	-/—	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 February 2000	15/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted]B 99/02970

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 03437 A (UNIV LIEGE) 5 April 1990 (1990-04-05) claims; figure 5; examples	1-8,11
A	WO 96 06627 A (UNIV TULANE) 7 March 1996 (1996-03-07) page 13, line 3 -page 14, line 22; claims	1-8,11
X	WO 85 02611 A (SCRIPPS CLINIC RES) 20 June 1985 (1985-06-20) claim 8	1-8,11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/02970

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9529701 A	09-11-1995	IL 109519 A	12-03-1999
		CA 2189469 A	09-11-1995
		EP 0759779 A	05-03-1997
WO 9520657 A	03-08-1995	AU 1532795 A	15-08-1995
		CA 2180726 A	03-08-1995
		EP 0738325 A	23-10-1996
EP 0095426 A	30-11-1983	FR 2527445 A	02-12-1983
DE 3430894 A	14-03-1985	IL 69558 A	30-06-1988
		FR 2550943 A	01-03-1985
		GB 2145419 A,B	27-03-1985
		US 4751064 A	14-06-1988
WO 9003437 A	05-04-1990	FR 2636842 A	30-03-1990
		AT 123525 T	15-06-1995
		DE 68923006 D	13-07-1995
		EP 0445128 A	11-09-1991
WO 9606627 A	07-03-1996	AU 709779 B	09-09-1999
		AU 3233795 A	22-03-1996
		BR 9508633 A	30-09-1997
		CA 2198586 A	07-03-1996
		CZ 9700562 A	15-10-1997
		EP 0777490 A	11-06-1997
		FI 970799 A	24-04-1997
		HU 77869 A	28-09-1998
		JP 10505059 T	19-05-1998
		NO 970993 A	25-04-1997
		NZ 291262 A	25-02-1999
		PL 318930 A	21-07-1997
		ZA 9506412 A	11-03-1996
WO 8502611 A	20-06-1985	US 4603049 A	29-07-1986
		AT 39700 T	15-01-1989
		AU 572821 B	19-05-1988
		AU 2436884 A	02-08-1984
		AU 3747185 A	26-06-1985
		DK 364685 A	09-08-1985
		DK 421584 A	03-09-1984
		EP 0117367 A	05-09-1984
		EP 0165307 A	27-12-1985
		ES 528649 A	01-05-1985
		FI 843451 A	03-09-1984
		FI 853082 A	12-08-1985
		GR 79805 A	31-10-1984
		JP 61500664 T	10-04-1986
		NO 843464 A	31-08-1984
		NO 853153 A	13-09-1985
		PH 23161 A	19-05-1989
		PT 77910 A,B	01-01-1984
		US 4758655 A	19-07-1988
		WO 8402700 A	19-07-1984
		US 4886663 A	12-12-1989
		ZA 8309512 A	29-08-1984

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of the Authority may be indicated by the applicant on the line below.

IPEA/ EPO

PCT

DEMAND

CHAPTER II

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: P007438WOCTH

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/GB99/02970	7 Sep 1999	7 Sep 1998

Title of invention: SUBSTANCE

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

University of Bristol
Senate House
Tyndall Avenue
Clifton
Bristol BS8 1TH
GB

Telephone No.:

Facsimile No.:

Teleprinter No.:

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

WILLIAMS, Neil Andrew
16 The Court
Old Coach Road
Cross, Axbridge
Somerset, BS26 2EF
United Kingdom

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HIRST, Timothy Raymond
30 Albert Road
Clevedon
North Somerset
BS21 7RR
United Kingdom

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

☐ Further applicants are indicated on a continuation sheet.

International application No. PCT/GB99/02970

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☑ agent ☐ common representative

and ☑ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HARDING, Charles Thomas
D Young & Co
21 New Fetter Lane
London
EC4A 1DA
United Kingdom

Telephone No.: +44 23 8064 4816

Facsimile No.: +44 23 8022 4262

Teleprinter No.: 477667 YOUNGS G

☐ **Address for Correspondence**: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments: *

1. The applicant wishes the international preliminary examination **to start on the basis of:**

☑ the international application as originally filed

the description
- ☐ as originally filed
- ☐ as amended under Article 34

the claims
- ☐ as originally filed
- ☐ as amended under Article 19 (together with any accompanying statement)
- ☐ as amended under Article 34

the drawings
- ☐ as originally filed
- ☐ as amended under Article 34

2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. ☐ The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired).*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

- ☑ which is the language in which the international application was filed.
- ☐ which is the language of a translation furnished for the purposes of international search.
- ☐ which is the language of publication of the international application.
- ☐ which is the language of translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby **elects all eligible States** *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

International application No.
PCT/GB99/02970

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:

			For International Preliminary Examining Authority use only	
			received	not received
1.	translation of international application	: sheets	☐	☐
2.	amendments under Article 34	: sheets	☐	☐
3.	copy (or, where required, translation) of amendments under Article 19	: sheets	☐	☐
4.	copy (or, where required, translation) of statement under Article 19	: sheets	☐	☐
5.	letter	: sheets	☐	☐
6.	other *(specify)*	: sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☑ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ nucleotide and or amino acid sequence listing in computer readable form
6. ☑ other *(specify)*: Letter

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

C T HARDING - Authorised Agent

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.

4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.

5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

See Notes to the demand form

CHAPTER II

PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No.	PCT/GB99/02970
Applicant's or agent's file reference	P007438WOCTH

For International Preliminary Examining Authority use only

Date stamp of the IPEA

Applicant

UNIVERSITY OF BRISTOL

Calculation of prescribed fees

1.	Preliminary examination fee	EUR 1,533	P
2.	Handling fee *(Applicants from certain states are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)*	EUR 147	H
3.	Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	EUR 1,680 TOTAL	

Mode of Payment

- [x] authorization to charge deposit account with the IPEA (see below)
- [ ] cash
- [ ] cheque
- [ ] revenue stamps
- [ ] postal money order
- [ ] coupons
- [ ] bank draft
- [ ] other *(specify)*:

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA/ EPO

- [x] is hereby authorized to charge the total fees indicated above to my deposit account.
- [x] *(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

28050042	28 Mar 2000		Charles Harding
Deposit Account Number	Date *(day/month/year)*	Signature	

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
HARDING, C.
D. YOUNG & CO.
21 New Fetter Lane
London EC4A 1DA
GRANDE BRETAGNE

MONEY £
ORDER
DIARY 24-10-00
RECD 26 JUL 2000
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ENTRY
FOR DS CTH
Records notes

SOUTHAMPTON
26 JUL 2000

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	24.07.00
Applicant's or agent's file reference: P007438WOCTH	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/02970	07/09/1999	07/09/1998

International Patent Classification (IPC) or both national classification and IPC
C07K14/28

Applicant
UNIVERSITY OF BRISTOL et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☒	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 07/01/2001.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner
Mundel, C

Formalities officer (incl. extension of time limits)
Faux, K
Telephone No. +49 89 2399 8062

WRITTEN OPINION

International application No. PCT/GB99/02970

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-45 as originally filed

Claims, No.:

1-11 as originally filed

Drawings, sheets:

1/8-8/8 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

II. Priority

1. ☐ This opinion has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application,

☒ claims Nos. 10,

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 10 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-9 and 11 (NO)
Inventive step (IS)	Claims	1-9 and 11 (NO)
Industrial applicability (IA)	Claims	11 (See Citations and explanations)

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item I
Basis of the opinion

A sequence listing has been filed with the present application on the date of 11.10.99. This listing contains SEQ ID NO:1 to 5 (page 1).

Re Item II
Priority

The priority document of the present application was not available at the time where this preliminary opinion has been drafted. The present analysis is based on the hypothesis that all the claims have a priority right corresponding to the date of filing of the priority document 07.09.98.

Re Item III
Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claim 10 lacks clarity and is not supported by the description (see points VIII-5 and VIII-6). Therefore, a meaningful evaluation regarding novelty, inventive step, and industrial applicability can not be carried out.

Re Item V
Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. **Reference is made to the following documents :**

D1: WO 95 29701 A (YEDA RES & DEV ;MIRELMAN DAVID (IL); MARKS ROBERT S (IL); SELA MIC) 9 November 1995 (1995-11-09)
D2: WO 95 20657 A (GX BIOSYSTEMS AS ;SOKURENKO EVGENI VENIAMINOVIC (US); HASTY DAVID) 3 August 1995 (1995-08-03)
D3: EP-A-0 095 426 (CENTRE NAT RECH SCIENT ;PASTEUR INSTITUT (FR)) 30 November 1983 (1983-11-30)
D4: DE 34 30 894 A (YEDA RES & DEV) 14 March 1985 (1985-03-14)

D5: WO 85 02611 A (SCRIPPS CLINIC RES) 20 June 1985 (1985-06-20)

2. The present application discloses the identification of an amino acid sequence which is important for the role of the CtxB toxin in triggering the depletion of CD8+ T-cells, in inducing a potent anti-EtxB response and to act as mucosal adjuvant However, the claims are directed to a substance comprising an amino acid comprising the sequence EVPGSQHI for use in medicine and more particularly as immunomodulator, adjuvant or inhibitor for toxin-induced diarrhoea. The claims also refer to a pharmaceutical composition comprising said substance, to the use of said substance in the manufacture of medicament, to a method for determining agents capable of interacting with "or affecting" said substance and to an agent identified by said method, and to a method of treatment comprising administering said substance to a subject.

3. Lack of novelty and inventive step; articles 33(2) and 33(3) PCT.

3.1 The document D1 discloses conjugates of an antigen selected from the group of a toxin or a fragment thereof, a toxoid and/or an adherence antigen derived from an infecting agent covalently bound to an inert carrier (Abstract, lines 1-3). Said conjugates are for use in **vaccines** for oral immunization against infecting agents (Abstract, lines 3-4). One of the antigen disclosed in D1 is the **peptide CTP3** consisting of the amino acid 50-64 of the cholera toxin B subunit chain. This peptide **comprises** the sequence EVPGSQHI (= SEQ ID NO:2) (p. 3, lines 14-19). D1 also states that a fragments from toxin from other enteric pathogens like Escherichia coli-LT (=EtxB) can be used. An experiment shows that the colostrum of female rabbits immunized with the conjugate silica-cholera toxin B subunit derived synthetic peptide CTP3 contains IgA directed against the native cholera toxin (p. 12, lines 9-17). A ELISA test in order to detect antibodies raised against two conjugates : Si-TGB-CTP3 and TGB-CTP3 - which both comprise the peptide CTP3 **linked to the thyroglobulin** - is also disclosed (p. 22, lines 10-23).

A solution containing the CTP3 peptide is considered as fulfilling the definition of claim 1 for the following reasons :

- Due to its small size (15 amino acids) and due to its localization in the

sequence of the cholera toxin B subunit sequence (AAs 50-64), the polypeptide CTP3 obviously has no GM1-binding activity.

- Antibodies directed against the CTP3 peptide can also recognize the native cholera toxin. Due to the lack of clarity mentioned in point VIII-1 and more especially point VIII-1 (v), the CTP3 peptide can be considered as being "capable of acting in a manner that is the same or is similar to CtxB" at least as far as the antigenicity is concerned.
- Since the CTP3 peptide contains the sequence shown in SEQ ID NO:2 which, according to the present application, plays an essential role in triggering the depletion of CD8+ T-cells (Example 1, p. 41-42), is required to induce a potent anti-EtxB response (Example 3, p.43) and is necessary for the toxin B-subunit to act as mucosal adjuvant (Example 4, p. 44), said peptide will, **per se**, have all those activities.

Thus, claims 1 and 7 can not be considered as new or inventive in the sense of articles 33(2) and 33(3) PCT.

Moreover, since the use of the CTP3 peptide as a vaccine has already been disclosed, the subject-matter of claims 2-5 (which are considered as first medical use by the European Patent Office) and 11 can not be considered as new or inventive in the sense of articles 33(2) and 33(3) PCT.

Furthermore, the thyroglobulin which is linked to the CTP3 peptide in the conjugates disclosed in D1 is considered as an additional antigen. Thus, the subject-matter of claim 6 can not be considered as new or inventive (articles 33(2) and 33(3) PCT).

Claim 8 of the present application is considered as a second medical use claim by the European Patent Office. Due to the clarity problem mentioned in point VIII-4, the vaccine against the cholera toxin containing the CTP3 peptide disclosed in D1 fit the definition of a medicament according to claim 8. Thus, claim 8 can not be considered as novel or inventive (articles 33(2) and 33(3) PCT).

The ELISA test used to determine if IgA (=agent) will bind to the conjugates comprising the CTP3 peptide (=substance according to any one of claims 1

to 7) fit the definition of the assay method disclosed in claim 9. Thus, the subject-matter of claim 9 can not be considered as new or inventive in the sense of article 33(2) and 33(3) PCT (see also point VIII-5).

3.2 The documents D2-D5 are also considered as relevant for the evaluation of the novelty and inventiveness of the claims and will be discussed briefly :

D2 refers, inter alia, to fusion proteins containing a neutralizing epitope of the cholera toxin B chain (which is the same as the CTP3 peptide of D1) inserted in two different positions in the FimH adhesin of type 1 fimbriae. The binding of anti-CtxB antibodies to the fusion protein has been tested. This document deprives claims 1, 6, 7 and 9 of novelty and inventiveness for the same reasons as those disclosed in point V-3.1 above.

D3 refers to new medicaments comprising at least one sequence of the cholera toxin B subunit, inter alia the sequence 50-75 which comprises the sequence EVPGSQHI. Said sequence has been used for the manufacture of a vaccine against cholera and other human and animal infections due to Escherichia coli enterotoxin LT (=EtxB). The binding of anti-CtxB antibodies to the fragment 50-75 has also been tested. For the reasons mentioned in point 3.1 above, claims 1-9 and 11 can not be considered as new or inventive over the teaching of D3.

D4 discloses, inter alia, the use of the CTP3 peptide as a vaccine against cholera and heat-labile E.coli toxin. The teaching of this document is almost the same as document D1. Thus claims 1-9 and 11 can not be considered as novel or inventive over the teaching of D4.

D5 discloses the use of peptides containing 10 to 35 amino acids residues corresponding the amino acids 35 to 95 from the B-subunit of the heat-labile enterotoxin of Escherichia coli - some of which contain the sequence EVPGSQHI (p. 75-77) - in polymers as the active ingredient of a vaccine for protection against infection by heat-labile enterotoxin-producing bacteria. The use of said peptides coupled to carriers is also disclosed. Therefore, claims 1-9 and 11 can not be considered to be novel or inventive over the teaching of D5 (see point 3.1 for explanations).

4. Industrial applicability; article 33(4) PCT.

Claim 11 refers to a method of treatment of the human or animal body.
For the assessment of the present claim 11 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII
Certain observations on the international application

Lack of clarity; article 6 PCT.

1. Claim 1 of the present application lacks clarity for the following reasons :

   (i) The use of the vague term "substance" renders the scope of the claim unclear since it can include lots of different components in addition to the amino acid sequence comprising the sequence shown in SEQ ID NO:2.

   (ii) Claim 1 refers to a "variant", "homologue", "derivative" or "mimetic" of an amino acid sequence. The use of these terms renders the scope of the claim unclear since there is no clear definition of what such a variant, homologue,

derivative or mimetic should be.
Moreover, it is not clear if these terms apply to the amino acid sequence shown in SEQ ID NO:2 or to the amino acid sequence comprising said sequence. In this written opinion, it has been assumed that said terms refer to the amino acid sequence shown in SEQ ID NO:2 which represents the only technical feature of claim 1.
Therefore, the IPEA is the opinion that a protein comprising an amino acid sequence differing from the sequence disclosed in SEQ ID NO:2 by one or even more amino acids can still be considered as **comprising** a "variant", "homologue" or "derivative" of said sequence. Some well-known proteins probably fit this definition.

(iii) It is not clear what is meant by "fragment" of an amino acid sequence of 8 amino acids. The attention of the applicant is drawn to the fact that even a single amino acid could be reasonably considered as a fragment of a 8 amino acid sequence. Thus, every protein can be considered as comprising a fragment of SEQ ID NO:2 and lots of well-known proteins contain at least 2 or 3 consecutive amino acids of SEQ ID NO:2.

(iv) Due to the clarity problems mentioned in points 1 (i), (ii) and (iii) above, the only distinction between the substance of the present application and well-known proteins - like, for example, the fragments of the EtxB and CtxB toxins amino acid sequences disclosed in D1-D5 - is the fact that the substance of claim 1 is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB, but does not exhibit GM-1 binding activity, i.e. by the result to be achieved.
According to the PCT Gazette of the 29.10.98 "PCT International Preliminary Examination Guidelines", Chapter III-4.7 : "The area defined by the claims must be as precise as the invention allows. As a general rule, claims which attempt to define the invention, or a feature thereof, by a result to be achieved should be objected to".
The substance of claim 1 should be described in terms of the technical features - for example specific amino acid sequences - which cause the substance of claim 1 to be capable of acting in a manner that is the same as or similar to EtxB and/or CtxB without exhibiting GM-1 binding activity.

(v) Claim 1 refers to a substance which is "capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB". This wording renders the scope of the claim unclear since there is no clear definition in the present application, and especially in the claims, of which activities of EtxB and CtxB are meant.
Moreover, the addition of the wording "capable of acting in a manner that is the same as **or is similar to** EtxB and/or CtxB" renders the scope of the claim even more unclear.

(vi) Moreover, the attention of the applicant is drawn to the fact that, in the present application, there is **no example** of a substance fulfilling the requirements of claim 1 since the **only** substance comprising an amino acid sequence **comprising** the sequence disclosed in SEQ ID NO:2 and which **does not exhibit** a GM-1 binding activity is the EtxB(G33D) mutant which **does not cause** a depletion of $CD8^+$ T-cells (Example 1) and which **does not**, therefore, act "in a manner that is the same as or is similar to EtxB". Thus, there is no support in the present application that a substance comprising an amino acid sequence comprising the sequence disclosed in SEQ ID NO:2 and which **does not have** a GM-1 binding activity **could retain** the ability to act "in a manner that is the same as or is similar to EtxB and/or CtxB" (article 5 PCT).

2. As a general remark about the different uses of the substance of claim 1, the attention of the applicant is drawn to the fact that, since there is no example of a substance fulfilling the definition of claim 1 in the present application, there are also no evidences that such a substance could be used as an immunomodulator, an adjuvant, an inhibitor of toxin-induced diarrhoea or could be used in the manufacture of a medicament for the treatment and/or the prevention and/or the modulation of a disease and/or condition associated with an immune disorder and/or a toxin induced diarrhoea disease. Thus, the different uses claimed for the substance of the present application are not considered as being supported by the description (article 5 PCT).

3. Claim 3 refers to the use of the substance of claim 1 as an immunomodulator. The wording of this claim is unclear since there is no clear definition of what is meant

by **"immunomodulator"**. In the broad sense of this term, each antigen can be considered as being an immunomodulator since it promotes the expansion of the pool of T and B-cells recognizing this specific antigen.

4. Claim 8 is unclear for the following reasons :

   (i) The attention of the applicant is drawn to the fact that the term "use" is redundant : use ... for use in the manufacture.

   (ii) There is no clear definition of what "modulating a disease" should be.

   (iii) There is no clear definition of what is meant by an "immune disorder".

   (iv) It is not clear if the wording "associated with an immune disorder" refers only to the term "condition" or also to the term "disease".

5. Claim 9 refers to an assay method for determining agents that are capable of interacting with and/or affecting the substance according to any of claims 1 to 7. The wording if said claim is unclear for the following reasons :

   (i) There is no clear definition of what "affecting the substance" should mean.

   (ii) Since the substance is not limited to an amino acid sequence comprising the sequence shown in SEQ ID NO:2 but can also include almost any other compounds (see point VIII-1 (i)), the assay method of claim 9 will not be limited to determine compounds interacting with or "affecting" the amino acid sequence comprising the sequence shown in SEQ ID NO:2 but will also include the detection of **any change in any of the compounds** included in the substance of claim 1. Lots of the methods encompassed by the present wording of claim 9 are well-known and will deprive claim 9 of novelty.

6. Claim 10 refers to an agent identified by the assay method according to claim 9. Due to the clarity problem mentioned in point VIII-5 above, the IPEA is the opinion that most of the compounds encompassed by said claim will be well-known compounds.

Even if claim 9 should be restricted to the detection of compounds interacting with and/or affecting the amino acid sequence comprised in the substance of claim 1, the IPEA is the opinion that claim 10 would still be unclear since the agents of said claim are **not** characterized by any **technical features**. Moreover, there is no description in the present application of what such an agent should be, thus the IPEA is the opinion that the agents claimed are not supported by the description of the present application (article 5 PCT).

7. Claim 11 is unclear for the following reasons :

(i) There is no clear definition of what a "condition associated with an immune disorder and/or a toxin mediated disorder" should be.

(ii) It is not clear what is meant by "**modulation** of a disease and/or condition associated with an immune disorder and/or a toxin mediated disorder".

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HARDING, C.
D. YOUNG & CO.
21 New Fetter Lane
London EC4A 1DA
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	05.12.2000
Applicant's or agent's file reference P007438WOCTH	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/02970	07/09/1999	07/09/1998

Applicant
UNIVERSITY OF BRISTOL et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P007438WOCTH		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/02970	07/09/1999	07/09/1998
International Patent Classification (IPC) or national classification and IPC C07K14/28		
Applicant UNIVERSITY OF BRISTOL et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 13 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
28/03/2000	05.12.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Mundel, C Telephone No. +49 89 2399 7314

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/02970

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-45 as originally filed

Claims, No.:

1-11 as originally filed

Drawings, sheets:

1/8-8/8 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
   see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 10.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 10 are so unclear that no meaningful opinion could be formed (*specify*):
see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion

could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-9 and 11
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-9 and 11
Industrial applicability (IA)	Yes:	Claims	1-9
	No:	Claims	11 (See Citations and explanations)

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I

Basis of the opinion

A sequence listing has been filed with the present application on the date of 11.10.99. This listing contains SEQ ID NO:1 to 5 (page 1).

Re Item II

Priority

The priority document of the present application was not available at the time where this preliminary opinion has been drafted. The present analysis is based on the hypothesis that all the claims have a priority right corresponding to the date of filing of the priority document 07.09.98.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claim 10 lacks clarity and is not supported by the description (see points VIII-5 and VIII-6). Therefore, a meaningful evaluation regarding novelty, inventive step, and industrial applicability can not be carried out.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. **Reference is made to the following documents :**

D1: WO 95 29701 A (YEDA RES & DEV ;MIRELMAN DAVID (IL); MARKS ROBERT S (IL); SELA MIC) 9 November 1995 (1995-11-09)

D2: WO 95 20657 A (GX BIOSYSTEMS AS ;SOKURENKO EVGENI VENIAMINOVIC (US); HASTY DAVID) 3 August 1995 (1995-08-03)

D3: EP-A-0 095 426 (CENTRE NAT RECH SCIENT ;PASTEUR INSTITUT (FR)) 30 November 1983 (1983-11-30)

D4: DE 34 30 894 A (YEDA RES & DEV) 14 March 1985 (1985-03-14)

D5: WO 85 02611 A (SCRIPPS CLINIC RES) 20 June 1985 (1985-06-20)

2. The present application discloses the identification of an amino acid sequence which is important for the role of the CtxB toxin in triggering the depletion of CD8+ T-cells, in inducing a potent anti-EtxB response and to act as mucosal adjuvant However, the claims are directed to a substance comprising an amino acid comprising the sequence EVPGSQHI for use in medicine and more particularly as immunomodulator, adjuvant or inhibitor for toxin-induced diarrhoea. The claims also refer to a pharmaceutical composition comprising said substance, to the use of said substance in the manufacture of medicament, to a method for determining agents capable of interacting with "or affecting" said substance and to an agent identified by said method, and to a method of treatment comprising administering said substance to a subject.

3. Lack of novelty and inventive step; articles 33(2) and 33(3) PCT.

3.1 The document D1 discloses conjugates of an antigen selected from the group of a toxin or a fragment thereof, a toxoid and/or an adherence antigen derived from an infecting agent covalently bound to an inert carrier (Abstract, lines 1-3). Said conjugates are for use in **vaccines** for oral immunization against infecting agents (Abstract, lines 3-4). One of the antigen disclosed in D1 is the **peptide CTP3** consisting of the amino acid 50-64 of the cholera toxin B subunit chain. This peptide **comprises** the sequence EVPGSQHI (= SEQ ID NO:2) (p. 3, lines 14-19). D1 also states that a fragments from toxin from other enteric pathogens like Escherichia coli-LT (=EtxB) can be used. An experiment shows that the colostrum of female rabbits immunized with the conjugate silica-cholera toxin B subunit derived synthetic peptide CTP3 contains IgA directed against the native cholera toxin (p. 12, lines 9-17). A ELISA test in order to detect antibodies raised against two conjugates : Si-TGB-CTP3 and TGB-CTP3 - which both comprise the peptide CTP3 **linked to the thyroglobulin** - is also disclosed (p. 22, lines 10-23).

A solution containing the CTP3 peptide is considered as fulfilling the definition of claim 1 for the following reasons :

- Due to its small size (15 amino acids) and due to its localization in the

sequence of the cholera toxin B subunit sequence (AAs 50-64), the polypeptide CTP3 obviously has no GM1-binding activity.

- Antibodies directed against the CTP3 peptide can also recognize the native cholera toxin. Due to the lack of clarity mentioned in point VIII-1 and more especially point VIII-1 (v), the CTP3 peptide can be considered as being "capable of acting in a manner that is the same or is similar to CtxB" at least as far as the antigenicity is concerned.
- Since the CTP3 peptide contains the sequence shown in SEQ ID NO:2 which, according to the present application, plays an essential role in triggering the depletion of CD8+ T-cells (Example 1, p. 41-42), is required to induce a potent anti-EtxB response (Example 3, p.43) and is necessary for the toxin B-subunit to act as mucosal adjuvant (Example 4, p. 44), said peptide will, **per se**, have all those activities.

Thus, claims 1 and 7 can not be considered as new or inventive in the sense of articles 33(2) and 33(3) PCT.

Moreover, since the use of the CTP3 peptide as a vaccine has already been disclosed, the subject-matter of claims 2-5 (which are considered as first medical use by the European Patent Office) and 11 can not be considered as new or inventive in the sense of articles 33(2) and 33(3) PCT.

Furthermore, the thyroglobulin which is linked to the CTP3 peptide in the conjugates disclosed in D1 is considered as an additional antigen. Thus, the subject-matter of claim 6 can not be considered as new or inventive (articles 33(2) and 33(3) PCT).

Claim 8 of the present application is considered as a second medical use claim by the European Patent Office. Due to the clarity problem mentioned in point VIII-4, the vaccine against the cholera toxin containing the CTP3 peptide disclosed in D1 fit the definition of a medicament according to claim 8. Thus, claim 8 can not be considered as novel or inventive (articles 33(2) and 33(3) PCT).

The ELISA test used to determine if IgA (=agent) will bind to the conjugates comprising the CTP3 peptide (=substance according to any one of claims 1

to 7) fit the definition of the assay method disclosed in claim 9. Thus, the subject-matter of claim 9 can not be considered as new or inventive in the sense of article 33(2) and 33(3) PCT (see also point VIII-5).

3.2 The documents D2-D5 are also considered as relevant for the evaluation of the novelty and inventiveness of the claims and will be discussed briefly :

D2 refers, inter alia, to fusion proteins containing a neutralizing epitope of the cholera toxin B chain (which is the same as the CTP3 peptide of D1) inserted in two different positions in the FimH adhesin of type 1 fimbriae. The binding of anti-CtxB antibodies to the fusion protein has been tested. This document deprives claims 1, 6, 7 and 9 of novelty and inventiveness for the same reasons as those disclosed in point V-3.1 above.

D3 refers to new medicaments comprising at least one sequence of the cholera toxin B subunit, inter alia the sequence 50-75 which comprises the sequence EVPGSQHI. Said sequence has been used for the manufacture of a vaccine against cholera and other human and animal infections due to Escherichia coli enterotoxin LT (=EtxB). The binding of anti-CtxB antibodies to the fragment 50-75 has also been tested. For the reasons mentioned in point 3.1 above, claims 1-9 and 11 can not be considered as new or inventive over the teaching of D3.

D4 discloses, inter alia, the use of the CTP3 peptide as a vaccine against cholera and heat-labile E.coli toxin. The teaching of this document is almost the same as document D1. Thus claims 1-9 and 11 can not be considered as novel or inventive over the teaching of D4.

D5 discloses the use of peptides containing 10 to 35 amino acids residues corresponding the amino acids 35 to 95 from the B-subunit of the heat-labile enterotoxin of Escherichia coli - some of which contain the sequence EVPGSQHI (p. 75-77) - in polymers as the active ingredient of a vaccine for protection against infection by heat-labile enterotoxin-producing bacteria. The use of said peptides coupled to carriers is also disclosed. Therefore, claims 1-9 and 11 can not be considered to be novel or inventive over the teaching of D5 (see point 3.1 for explanations).

4. Industrial applicability; article 33(4) PCT.

Claim 11 refers to a method of treatment of the human or animal body.
For the assessment of the present claim 11 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII
Certain observations on the international application

Lack of clarity; article 6 PCT.

1. Claim 1 of the present application lacks clarity for the following reasons :

   (i) The use of the vague term "substance" renders the scope of the claim unclear since it can include lots of different components in addition to the amino acid sequence comprising the sequence shown in SEQ ID NO:2.

   (ii) Claim 1 refers to a "variant", "homologue", "derivative" or "mimetic" of an amino acid sequence. The use of these terms renders the scope of the claim unclear since there is no clear definition of what such a variant, homologue,

derivative or mimetic should be.
Moreover, it is not clear if these terms apply to the amino acid sequence shown in SEQ ID NO:2 or to the amino acid sequence comprising said sequence. In this written opinion, it has been assumed that said terms refer to the amino acid sequence shown in SEQ ID NO:2 which represents the only technical feature of claim 1.
Therefore, the IPEA considers that a protein comprising an amino acid sequence differing from the sequence disclosed in SEQ ID NO:2 by one or even more amino acids can still be considered as **comprising** a "variant", "homologue" or "derivative" of said sequence. Some well-known proteins probably fit this definition.

(iii) It is not clear what is meant by "fragment" of an amino acid sequence of 8 amino acids. The attention of the applicant is drawn to the fact that even a single amino acid could be reasonably considered as a fragment of a 8 amino acid sequence. Thus, every protein can be considered as comprising a fragment of SEQ ID NO:2 and lots of well-known proteins contain at least 2 or 3 consecutive amino acids of SEQ ID NO:2.

(iv) Due to the clarity problems mentioned in points 1 (i), (ii) and (iii) above, the only distinction between the substance of the present application and well-known proteins - like, for example, the fragments of the EtxB and CtxB toxins amino acid sequences disclosed in D1-D5 - is the fact that the substance of claim 1 is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB, but does not exhibit GM-1 binding activity, i.e. **by the result to be achieved.**
According to the PCT Gazette of the 29.10.98 "PCT International Preliminary Examination Guidelines", Chapter III-4.7 : "The area defined by the claims must be as precise as the invention allows. As a general rule, claims which attempt to define the invention, or a feature thereof, by a result to be achieved should be objected to".
The substance of claim 1 should be described in terms of the technical features - for example specific amino acid sequences - which cause the substance of claim 1 to be capable of acting in a manner that is the same as or similar to EtxB and/or CtxB without exhibiting GM-1 binding activity.

(v) Claim 1 refers to a substance which is "capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB". This wording renders the scope of the claim unclear since there is no clear definition in the present application, and especially in the claims, of which activities of EtxB and CtxB are meant.
Moreover, the addition of the wording "capable of acting in a manner that is the same as **or is similar to** EtxB and/or CtxB" renders the scope of the claim even more unclear.

(vi) Moreover, the attention of the applicant is drawn to the fact that, in the present application, there is **no example** of a substance fulfilling the requirements of claim 1 since the **only** substance comprising an amino acid sequence **comprising** the sequence disclosed in SEQ ID NO:2 and which **does not exhibit** a GM-1 binding activity is the EtxB(G33D) mutant which **does not cause** a depletion of $CD8^+$ T-cells (Example 1) and which **does not**, therefore, act "in a manner that is the same as or is similar to EtxB". Thus, there is no support in the present application that a substance comprising an amino acid sequence comprising the sequence disclosed in SEQ ID NO:2 and which **does not have** a GM-1 binding activity **could retain** the ability to act "in a manner that is the same as or is similar to EtxB and/or CtxB" (article 5 PCT).

2. As a general remark about the different uses of the substance of claim 1, the attention of the applicant is drawn to the fact that, since there is no example of a substance fulfilling the definition of claim 1 in the present application, there are also no evidences that such a substance could be used as an immunomodulator, an adjuvant, an inhibitor of toxin-induced diarrhoea or could be used in the manufacture of a medicament for the treatment and/or the prevention and/or the modulation of a disease and/or condition associated with an immune disorder and/or a toxin induced diarrhoea disease. Thus, the different uses claimed for the substance of the present application are not considered as being supported by the description (article 5 PCT).

3. Claim 3 refers to the use of the substance of claim 1 as an immunomodulator. The wording of this claim is unclear since there is no clear definition of what is meant

by "**immunomodulator**". In the broad sense of this term, each antigen can be considered as being an immunomodulator since it promotes the expansion of the pool of T and B-cells recognizing this specific antigen.

4. Claim 8 is unclear for the following reasons :

   (i) The attention of the applicant is drawn to the fact that the term "use" is redundant : use ... for use in the manufacture.

   (ii) There is no clear definition of what "modulating a disease" should be.

   (iii) There is no clear definition of what is meant by an "immune disorder".

   (iv) It is not clear if the wording "associated with an immune disorder" refers only to the term "condition" or also to the term "disease".

5. Claim 9 refers to an assay method for determining agents that are capable of interacting with and/or affecting the substance according to any of claims 1 to 7. The wording of said claim is unclear for the following reasons :

   (i) There is no clear definition of what "affecting the substance" should mean.

   (ii) Since the substance is not limited to an amino acid sequence comprising the sequence shown in SEQ ID NO:2 but can also include almost any other compounds (see point VIII-1 (i)), the assay method of claim 9 will not be limited to determine compounds interacting with or "affecting" the amino acid sequence comprising the sequence shown in SEQ ID NO:2 but will also include the detection of **any change in any of the compounds** included in the substance of claim 1. Lots of the methods encompassed by the present wording of claim 9 are well-known and will deprive claim 9 of novelty.

6. Claim 10 refers to an agent identified by the assay method according to claim 9. Due to the clarity problem mentioned in point VIII-5 above, the IPEA considers that most of the compounds encompassed by said claim will be well-known compounds.

Even if claim 9 should be restricted to the detection of compounds interacting with and/or affecting the amino acid sequence comprised in the substance of claim 1, the IPEA considers that claim 10 would still be unclear since the agents of said claim are **not** characterized by any **technical features**. Moreover, there is no description in the present application of what such an agent should be, thus the IPEA considers that the agents claimed are not supported by the description of the present application (article 5 PCT).

7. Claim 11 is unclear for the following reasons :

(i) There is no clear definition of what a "condition associated with an immune disorder and/or a toxin mediated disorder" should be.

(ii) It is not clear what is meant by "**modulation** of a disease and/or condition associated with an immune disorder and/or a toxin mediated disorder".

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference (if desired) (12 characters maximum) P/7438.WOCTH

Box No. I TITLE OF INVENTION

Substance

Box No. II APPLICANT

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

University of Bristol
Senate House
Tyndall Avenue
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Bristol BS8 1TH
GB

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State (i.e. country) of nationality: GB

State (i.e. country) of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☑ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

WILLIAMS, Neil Andrew
16 The Court
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United Kingdom

This person is:

☐ applicant only

☑ applicant and inventor

☐ inventor only (if this check-box is marked, do not fill in below)

State (i.e. country) of nationality: GB

State (i.e. country) of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☑ the United States of America only ☐ the States indicated in the Supplemental Box

☑ Further applicant and/or (further) inventors are indicated on a continuation sheet

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☑ agent ☐ common representative

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

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☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III — FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

HIRST, Timothy Raymond
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This person is:

- ☐ applicant only
- ☑ applicant and inventor
- ☐ inventor only (if this check-box is marked, do not fill in below)

State (that is, country) of nationality: GB

State (that is, country) of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☑ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:

- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only (if this check-box is marked, do not fill in below)

State (that is, country) of nationality:

State (that is, country) of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:

- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only (if this check-box is marked, do not fill in below)

State (that is, country) of nationality:

State (that is, country) of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:

- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only (if this check-box is marked, do not fill in below)

State (that is, country) of nationality:

State (that is, country) of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

Regional Patent

- ☑ AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☑ EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☑ EP European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein,CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☑ OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, please specify on dotted line)

National Patent (if other kind of protection or treatment desired, specify on dotted line):

- ☑ AL Albania
- ☑ AM Armenia
- ☑ AT Austria
- ☑ AU Australia
- ☑ AZ Azerbaijan
- ☑ BA Bosnia and Herzegovina
- ☑ BB Barbados
- ☑ BG Bulgaria
- ☑ BR Brazil
- ☑ BY Belarus
- ☑ CA Canada
- ☑ CH AND LI Switzerland and Liechtenstein
- ☑ CN China
- ☑ CU Cuba
- ☑ CZ Czech Republic
- ☑ DE Germany
- ☑ DK Denmark
- ☑ EE Estonia
- ☑ ES Spain
- ☑ FI Finland
- ☑ GB United Kingdom
- ☑ GD Grenada
- ☑ GE Georgia
- ☑ GH Ghana
- ☑ GM Gambia
- ☑ HR Croatia
- ☑ HU Hungary
- ☑ ID Indonesia
- ☑ IL Israel
- ☑ IN India
- ☑ IS Iceland
- ☑ JP Japan
- ☑ KE Kenya
- ☑ KG Kyrgyzstan
- ☑ KP Democratic People's Republic of Korea
- ☑ KR Republic of Korea
- ☑ KZ Kazakhstan
- ☑ LC Saint Lucia
- ☑ LK Sri Lanka
- ☑ LR Liberia
- ☑ LS Lesotho
- ☑ LT Lithuania
- ☑ LU Luxembourg
- ☑ LV Latvia
- ☑ MD Republic of Moldova
- ☑ MG Madagascar
- ☑ MK The former Yugoslav Republic of Macedonia
- ☑ MN Mongolia
- ☑ MW Malawi
- ☑ MX Mexico
- ☑ NO Norway
- ☑ NZ New Zealand
- ☑ PL Poland
- ☑ PT Portugal
- ☑ RO Romania
- ☑ RU Russian Federation
- ☑ SD Sudan
- ☑ SE Sweden
- ☑ SG Singapore
- ☑ SI Slovenia
- ☑ SK Slovakia
- ☑ SL Sierra Leone
- ☑ TJ Tajikistan
- ☑ TM Turkmenistan
- ☑ TR Turkey
- ☑ TT Trinidad and Tobago
- ☑ UA Ukraine
- ☑ UG Uganda
- ☑ US United States of America
- ☑ UZ Uzbekistan
- ☑ VN Viet Nam
- ☑ YU Yugoslavia
- ☑ ZW Zimbabwe

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after the issuance of this sheet:

- ☑ AE United Arab Emirates
- ☑ ZA South Africa
- ☑ CR Costa Rica DM Dominica TZ Tanzania

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

1. If, in any of the Boxes, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:

(i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;

(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;

(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;

(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;

(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "continuation" or "continuation-in-part": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;

(vi) if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;

(vii) if, in Box No. VI, the earlier application is an ARIPO application: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.

2. If, with regard to the precautionary designation statement contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.

3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.

Continuation of Box No. IV

PURVIS, William Michael Cameron
COTTER, Ivan John
PILCH, Adam John Michael
CRISP, David Norman
ROBINSON, Nigel Alexander Julian
HARRIS, Ian Richard
HARDING, Charles Thomas
TURNER, James Arthur
MALLALIEU, Catherine Louise
PRATT, Richard Wilson
PRICE, Paul Anthony King
HOLMES, Miles
HORNER, David Richard
MASCHIO, Antonio
NACHSHEN, Neil
POTTER, Julian
HAINES, Miles
MATHER, Belinda

Box No. VI PRIORITY CLAIM

☐ Further priority claims are indicated in the Supplemental Box

The priority of the following earlier application(s) is hereby claimed:

	Filing Date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application: * regional Office	international application: receiving Office
item (1)	7 Sep 1998 7/9/1998	9819484.8	UK		
item (2)					
item (3)					

☑ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s) : (1)

** Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number: Country (or regional Office):

Box No. VII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	:	5
description (excluding sequence listing part)	:	46
claims	:	2
abstract	:	1
drawings	:	8
sequence listing part of description	:	1
Total number of sheets	:	63

This international application is accompanied by the item(s) marked below:

1. ☐ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority documents(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☑ other *(specify):* Letter

Figure of the drawings **which should accompany the abstract:**

Language of filing of the international application:

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request)

C T HARDING

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA /
6. ☐ Transmittal of search copy delayed until search fee paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau: